INDIAN AGRICULTURAL
RESEARCH INSTITUTE, NEW DELHI.

JOURNAL

OF

BACTERIOLOGY

VOLUME 56

BIOCHEMICAL MUTANTS OF BACTERIAL VIRUSES[1]

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Two years ago T. F. Anderson (1945a) reported the remarkable discovery that certain strains of bacterial virus (T4 and T6) require organic substances as cofactors for the process of adsorption of the virus on the bacterial host strain B of *Escherichia coli*. Tryptophan in its L form is the substance most active in promoting adsorption of phage to bacterium, about 1 μg per ml being sufficient for maximum effect. Other substances, like phenylalanine and tyrosine, and several tryptophan analogues are also active, though only at higher concentrations (T. F. Anderson, 1945b, 1946). Anderson (1948) has shown that the function of the cofactor is to "activate" the phage particle reversibly, and that phage particles activated in this way will become deactivated in the absence of extraneous cofactor with a half-life of the order of one-half minute.

We wish to report some observations on the mutability of phage T4 with respect to cofactor requirements, and on certain features throwing additional light on the mechanism of cofactor action.

We were led to undertake these studies by the desire to have available a greater variety of phage mutants for genetic studies in bacterial viruses. Luria (1945) and Hershey (1946a,b) have previously described host range and plaque morphology mutants of phages, and we (Delbrück and Bailey, 1946) have reported on a peculiar transfer of genetic markers from one phage to another during mixed infections of bacteria with two different phages. Luria (1947) has recently analyzed a phenomenon of "reactivation" of irradiated phages in mixed infections and has interpreted his findings on the assumption of a similar transfer of genetic material from one phage particle to another. For further analysis of the mechanisms involved the availability of a greater variety of mutant types is clearly desirable. Anderson's discovery offered the hope of finding mutations with respect to biochemical requirements of the phage.

We have found three clearly distinct mutant types of T4, as follows: (1) a type requiring no cofactor; (2) a type requiring tryptophan or similar substances; and (3) a type requiring tryptophan or similar substances and in addition requiring Ca^{++} ions. There appears to be some correlation between cofactor requirement and plaque morphology. Each mutant that does not require a cofactor gives small plaques with a sharper margin than those of the wild type.

We have also found that indole (and to a lesser extent skatole) inhibits the adsorption of the mutants requiring tryptophan for adsorption.

MATERIALS AND METHODS

Starting Material and Isolation of Mutants

Anderson noticed the cofactor requirement of phage strains T4 and T6 when plating these stocks on petri plates containing a synthetic medium agar.[2] On these plates the plaque counts of Anderson's T4 and T6 stocks were much lower than on parallel broth plates. In going through our stocks of T4 we found that stocks made with synthetic media gave very similar plaque counts on synthetic medium plates and on broth plates. Presumably during the growth of these stocks a selection of mutants of T4 not requiring cofactors had taken place. However, an old broth stock showed the Anderson phenomenon, a much lower plaque count on synthetic medium plates than on broth plates. Moreover the platings on broth showed three distinguishable types of plaque, viz.: (1) "f," wild type, plaques with fuzzy edge; (2) "s," plaques with very sharp edge (Hershey, 1946a,b); and (3) "r," plaques with clear halo (Hershey, 1946a,b). Several plaques of each type were picked and their contents plated out in parallel on F and on broth plates. Each of the three types bred true on subculture (except for small proportions of mutants). The s plaques gave the same plaque counts on F and on broth, whereas the fuzzy and the r types gave very low or zero plaque counts on F plates. From broth plates of these stocks s, f, and r plaques were picked again and broth stocks were made. When these stocks were tested for deficiencies again, the s stock was found nondeficient, and the wild-type stock and the r were found deficient.

Notation

We designate each stock obtained from a single plaque and grown in liquid medium with a serial number. Thus, the three stocks just mentioned will be called: T4,1 (s type, nondeficient); T4,2 (f type, deficient); and T4,3 (r type, deficient). Such stocks need not be pure stocks. They may contain an admixture of mutants that arose during the growth of the plaque and during the subsequent growth of the stock in liquid medium. In general this admixture of mutants will amount to at most a few per cent. Thus, T4,1 contains about 10 per cent wild-type plaques; T4,2 contains about 1 per cent r plaques; and T4,3 contains less than 0.1 per cent of mutants giving plaques that are not of the r type. In some cases, when a mutation occurred early during the growth of a plaque from which a stock is isolated, the stock may be not even approximately pure. The stock T4,5, for instance, isolated from a single plaque on a broth plate and grown in broth, contained 25 per cent particles adsorbable with tryptophan only, and 75 per cent particles requiring other cofactors besides tryptophan.

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[2] Composition of our synthetic medium (F medium), which is similar to that of T. F. Anderson (1945a); Na-lactate 10 g, NH_4Cl 1 g, KH_2PO_4 1.5 g, Na_2HPO_4 3.5 g, $MgSO_4$ 0.1 g, H_2O (redistilled) 1,000 ml.

Measurement of Adsorption

The degree to which adsorption takes place in a given mixture of bacteria and phage can be determined in several ways:

(1) The adsorbed phage can be sedimented with the bacteria to which it is adsorbed and the free phage determined by a plaque count of the supernatant. The adsorbed fraction is then determined as the difference between the total phage and the free phage. This method is rather inaccurate when adsorption is slight and in such a case does not tell more than that the fraction adsorbed was less than, say, 10 per cent of the total.

(2) T. F. Anderson (1945a) has found that in cofactor-requiring stocks the adsorbed fraction can be determined directly by plating on the basal medium. On such plates free phage particles will not form plaques, whereas phage particles that were adsorbed to bacteria at the time of plating will form plaques even though no cofactor is added to the plate. Presumably the infected bacteria upon lysis yield a proportion of phage particles that are *temporarily* adsorbable without the need of extraneous cofactors. The bacteria may contain cofactor substances in sufficiently high concentration to activate the phage particles before their release, and a few particles may infect other bacteria on the plate before becoming deactivated. This is a hypothetical mechanism; but, whatever the mechanism may be, the fact remains that the plaque count on the basal medium plates yields a fair measure of the adsorbed fraction of the phage particles. We will cite as an example one experiment in which both of these methods were used on the same set of adsorption tubes (exp. 1).

Experiment 1. Determination of adsorption by two methods

1. By plaque count of free phage (supernatant on broth plates)
2. By plaque count of adsorbed phage (basal medium plates)

	COFACTORS ADDED TO ADSORPTION TUBE			
	DL-Typtophan 0.2 mg/ml	DL-Phenylalanine 0.2 mg/ml	N-Z-case 4 mg/ml	None
	%	%	%	%
1	24	37	79	−6
2	21	32	73	0

The second method gives slightly lower values for the adsorbed fraction than the first one. This means that not every adsorbed particle forms a plaque on basal medium plates. It is necessary, therefore, to check the second method with the first method for each new stock under investigation. This has been done for each stock described in this paper. In some cases it was found that the second method gives values less than 50 per cent of those obtained by the first method. In such cases also the appearance of the plaques on F agar is very poor and the count uncertain and variable. Nevertheless the second method is a valuable one, particularly in cases of slight adsorption where the first method yields only qualitative results. For the measurements cited here the second

method gave at least 50 per cent of the adsorbed fraction. For each measurement cited we will indicate which of the two methods was used.

Measurement of Cofactor Activity

The function of the cofactors, according to T. F. Anderson (1948), is to "activate" the phage particles, i.e., to bring them into a state in which they can be adsorbed by the bacteria. The word "adsorption" is here used simply to designate the fact that phage particle and bacterium form a link that is not reversible by dilution or any other known treatment. It used to be thought that this linkage was analogous to the bonds formed between antigens and antibodies. Anderson's discovery of cofactor requirements points in a different direction. It may turn out that adsorption consists not merely in the formation of a static bond, controlled by secondary valencies, but involves enzymatic reactions. Whatever the mechanism, closer studies of the kinetics of cofactor-controlled activation should help to get a clearer picture of its nature.

Cofactor activity can be demonstrated and titrated by two methods that measure the adsorption obtainable under different conditions.

(1) *Activation prior to adsorption.* The cofactor is added to a suspension of phage particles, and the mixture is incubated to permit the reaction between phage and cofactor to come to equilibrium. The mixture is then diluted with a large volume of bacterial suspension and plated after adsorption. Since deactivation of the phage particles after dilution is very rapid, only a fraction of the activated particles will be adsorbed after dilution. The adsorbed fraction will depend on the concentration of bacteria in a predictable way. T. F. Anderson (1948) has made ingenious use of this method to prove that deactivation proceeds exponentially, like the decay of a radio-active substance. The method may also be used to determine the dependence on the concentration of cofactor of the active fraction of phage at equilibrium. In agreement with Anderson we find that the active fraction drops very suddenly to zero when the concentration of cofactor is lowered below a certain threshold, characteristic for each cofactor (exp. 2).

Experiment 2. Activation of phage by various concentrations of cofactor (L-tryptophan)

Phage and cofactor equilibrated for 30 minutes at 37 C, diluted 1:25 with bacterial suspension, plated on basal medium 5 minutes later.

L-TRYPTOPHAN	ADSORPTION (METHOD 2)
μg/ml	%
2	5
1	2.3
0.5	0.8
0.25	0.005
0.125	0.005
None	0.004

The active fraction drops by more than a factor of 100 when the concentration of cofactor is lowered from 0.5 μg per ml to 0.25 μg per ml.

(2) *Activation during adsorption.* The activity of a cofactor may also be demonstrated and titrated by adding the cofactor to the adsorption mixture, and by measuring the amount of adsorption obtained after a definite length of time. Under these conditions activation and deactivation of the phage particles and adsorption proceed simultaneously, and the amount of adsorption obtainable depends on the relative rates of these three processes. For this reason the measured adsorption is a more indirect measure of activation than that obtained by the first method. There are actually two further factors affecting the amount of adsorption obtainable by this method. First, the bacteria will metabolize the cofactor and change its concentration during the course of the experiment. Secondly, while metabolizing the cofactor, the bacteria may produce a substance that actively inhibits the activation of the phage particles by the cofactor. Each of these factors would have to be worked out quantitatively before activity measurements obtained by this method could be translated into terms of inter-action between phage particles and cofactor. This has not yet been accom-plished, and for this reason the activity measurements obtained by this method

TABLE 1

Relative activities of cofactors

COFACTOR	CONCENTRATION FOR HALF-MAXIMUM ADSORPTION
	µg/ml
L-Tryptophan....	1
DL-5-Methyl-tryptophan...	14
DL-Phenylalanine.....	18
L-Tyrosine...........................	170
DL-Norleucine......	340

will not be given in detail. Notwithstanding these complications, this method is a convenient one for a rapid survey of a large number of potential cofactors and for a rough comparison of their relative activities. When the concentration of a given cofactor is varied, a result is obtained similar to that with the first method, namely, a threshold concentration of cofactor at which a rapid drop in activity occurs. In table 1 the concentrations of different cofactors are given at which half-maximum adsorption is obtained.

The weak activities of tyrosine and of norleucine might be suspected as due to impurities. However, they were rechecked and confirmed with highly purified samples of different origins.

RESULTS

The Stock T4,5

For most of the earlier experiments reported in this paper the stock T4,5 was used. This stock had been assumed to be reasonably pure since (1) it was isolated from a single plaque and (2) since it exhibited no heterogeneity in plaque morphology when plated on broth plates.

However, during the course of these experiments it became quite apparent

that T4,5 is inhomogeneous with respect to the very character under investigation, viz., its cofactor requirement.   Only 25 per cent of the particles of this stock were adsorbable on bacteria in a synthetic medium in the presence of any amount of tryptophan.   It could be shown that this result was not due to any sort of kinetic equilibrium between adsorption and desorption, or activation and deactivation, but to a true inhomogeneity among the particles of the stock, and this inhomogeneity could be used to separate the stock into fractions of different adsorbability (exp. 3).

Experiment 3.   Fractionation of stock T4,5

	ADSORPTION (METHOD 2)
	%
Adsorb T4,5 on B in presence of 4 µg/ml L-tryptophan....	20
Adsorb supernatant on fresh B in presence of 4 µg/ml L-tryptophan.................	3.7

It is quite apparent from these experiments that the fraction that does not respond to the cofactor tryptophan must be adsorbable in the presence of some other, or some additional, cofactor, since this fraction is adsorbable in the presence of broth.   Further experiments, involving tests on the contents of individual plaques, showed that the character difference here encountered might be a hereditary character of sufficient stability to make worth while an attempt at obtaining pure stocks.   Consequently, suitable single plaque isolations were made, as indicated below, which led to stocks with the desired properties.

Preparation of Stocks with Different Cofactor Requirements

(1) A stock of particles adsorbable with tryptophan as the only supplement to F medium.

T4,5 was adsorbed on B in the presence of 4 µg per ml L-tryptophan, and plated on F plates.   Under these conditions the nonadsorbable fraction will be prevented from forming plaques.   A single plaque was picked into a suspension of bacteria in broth, incubated, and filtered after lysis.   This is stock T4,11.

(2) A stock of particles *not* adsorbable with tryptophan as the only supplement to F medium.

T4,5 was adsorbed on B in the presence of 4 µg per ml L-tryptophan, as above.   After adsorption the mixture was centrifuged and the *supernatant* plated on broth plates.   Adsorption and centrifugation removed the adsorbable fraction, and every plaque represents the progeny of a particle nonadsorbable with tryptophan alone.   One such plaque was picked into a suspension of bacteria in broth, incubated, and filtered after lysis.   This is stock T4,12.

The two stocks differ strikingly in their response to tryptophan.   T4,11 is as highly adsorbable with tryptophan as with broth, whereas T4,12 gives very low adsorption with tryptophan but high adsorption with broth.   The question

regarding the nature of the cofactor requirements of T4,12 arises, and this will be taken up next.

Identification of the Cofactor Requirements of T4,12

At first it was assumed that T4,12 requires as cofactor an organic compound different from tryptophan. A systematic search was undertaken using the customary techniques for identification of the growth factor requirements of microorganisms. An aliquot of the material to be assayed for cofactor activity was added to a mixture of bacteria and T4,12, and this mixture was plated after 10 minutes in parallel on F plates and on broth plates. High activity was shown by broth, N-Z-case, yeast extract, and acid-hydrolyzed yeast extract; very low activity was shown by acid-hydrolyzed casein with or without a supplement of tryptophan. Of the individual amino acids only tryptophan and phenylalanine at 25 μg per ml showed a slight activity. A mixture of the known vitamins plus acid-hydrolyzed nucleic acid was inactive.

TABLE 2

Properties of stocks T4,11 and T4,12

COFACTORS IN ADSORPTION TUBE	TITER	
	T4,11	T4, 12
	3×10^{10}	5.7×10^{10}
	Adsorption (method 2)	
	%	%
Broth................	90	80
4 μg/ml tryptophan....	87	4.3
Basal medium.......	0.001	0.001

Since these findings, as well as several others, did not give a clear lead to an organic compound, we suspected that an inorganic ion might be involved. Of these, the ions of Na, K, Mg, Cl, and phosphate could be excluded since they are present in our basal medium. Since calcium ions have been shown to affect several phage-bacterial systems, we suspected that here too calcium ion might be involved. This turned out to be correct, as may be seen from the results of experiment 4.

Experiment 4. Adsorption of T4,12 with varying amounts of L-tryptophan and CaCl₂

μg/ml L-TRYPTOPHAN	PLAQUE COUNTS ON BASAL MEDIUM PLATES			
	μg/ml CaCl₂			
	0	8	32	128
0	0	0	0	0
25	74	373	551	513
100	71	419	565	565

This experiment shows that in the presence of tryptophan, CaCl₂ very much increases the number of phage-bacterium complexes capable of plaque formation on F agar plates. Other tests showed that close to 100 per cent of the phage particles form plaques if the medium contains more then 10 μg per ml of tryptophan and of CaCl₂ each. About 30 per cent of the phage particles form plaques in the presence of 2 μg per ml of each of the cofactors.

Experiment 5 was designed to decide whether CaCl₂ is merely an activator of a phage-bacterium complex formed independently of it or whether it is necessary for the adsorption process. The experiment shows that Ca⁺⁺ is necessary for adsorption.

Experiment 5.   Adsorption of T4,12 with and without CaCl₂

Adsorption mixtures with and without calcium ions were set up in parallel, and free phage was measured after 10 minutes in the usual manner. Both adsorption mixtures contained 4 μg per ml L-tryptophan, and one of them contained 4 μg per ml CaCl₂.

IN ADSORPTION TUBE	ADSORPTION (METHOD 1)
	%
4 μg/ml L-tryptophan................... .	13
4 μg/ml L-tryptophan plus 4 μg/ml CaCl₂ ..	68

The requirement of T4,12 for Ca⁺⁺ is a specific one, in the sense that Ca⁺⁺ cannot be replaced by Mg⁺⁺ ion, since the minimal medium used in all the tests (F medium) contains 20 μg Mg⁺⁺ per ml. Also the Ca⁺⁺ requirement of T4,12 does not constitute a specialization of a general requirement for divalent cations, possibly present in all mutants. T4,11 was tested for adsorbability in the presence of L-tryptophan using a modified basal medium in which MgSO₄ was replaced by K₂SO₄. This medium contains no divalent cations, but their absence did not affect the adsorbability of T4,11. '

It is true that for many phages the adsorption process is dependent on the presence of electrolytes, and T4 with all the mutants here studied belongs in this group. In salt-free media no adsorption takes place. The salt requirement, however, is nonspecific with respect to the chemical nature of the ions and lies in a concentration range about a thousand times higher than the specific Ca⁺⁺ requirement of T4,12. The unspecific salt requirement presumably influences the electrostatic properties of the bacteria and of the phage particles.

Inhibition of Adsorption by Metabolic Products of Tryptophan

Tryptophan is a substrate actively metabolized by *E. coli*. Therefore, if tryptophan is incubated with bacteria, it will disappear in due course. It was thought that the cofactor activity of tryptophan might be used to follow quantitatively the progress of the disappearance of tryptophan. It turned out, however, that the bacteria produce from tryptophan a substance or substances that actively inhibit the adsorption of the cofactor-requiring phages (exp. 6).

Experiment 6. Inhibition of adsorption of T4,12 by preincubation of bacteria for 2 hours with 4 µg per ml L-tryptophan

A growing culture of B in F medium was divided into two equal portions, to one of which 4 µg per ml L-tryptophan were added, and both cultures were incubated for 2 hours. To both cultures were then added 4 µg per ml L-tryptophan, 4 µg per ml $CaCl_2$, and T4,12, and the adsorption of T4,12 on the bacteria was determined.

	ADSORPTION	
	METHOD 1	METHOD 2
	%	%
B preincubated with 4 µg/ml tryptophan................................	20	4
Control...........	80	75

It is clear from this experiment that bacteria preincubated with tryptophan will not adsorb T4,12 in the presence of more tryptophan and Ca^{++}. In experiment 7 various periods of preincubation with tryptophan were compared.

Experiment 7. Dependence of inhibition on time of preincubation with tryptophan

TIME OF PREINCUBATION MINUTES	ADSORPTION (METHOD 2)
	%
0	86
5	58
15	9
30	4.4
60	4.0
180	4.7

The inhibition created by preincubation with 4 µg per ml L-tryptophan is not overcome by adding to the adsorption tube 10 times this amount of L-tryptophan, nor by adding 40 µg per ml DL-phenylalanine (exp. 8).

Experiment 8. Inability of phenylalanine and of large amounts of tryptophan to overcome the inhibition created by preincubation with 4 µg per ml L-tryptophan

COFACTOR	ADSORPTION (METHOD 2)
	%
DL-Phenylalanine 40 µg/ml.............................	0.2
L-Tryptophan 40 µg/ml.................................	2

The dependence of the inhibition on the amount of tryptophan added at the beginning of the preincubation period was measured next. The duration of preincubation was kept constant at 60 minutes, and the amount of tryptophan added with the phage was also kept constant at 4 µg per ml (exp. 9).

Experiment 9. Dependence of inhibition on amount of tryptophan in preincubation tube

(Preincubation for 60 minutes. Four μg per ml L-tryptophan added to adsorption tube.)

L-TRYPTOPHAN IN PREINCUBATION TUBE	ADSORPTION (METHOD 2)
$\mu g/ml$	%
0	53
0.5	35
1	22
2	13
4	3
40	1

In a parallel experiment 80 μg per ml DL-phenylalanine were added to the preincubation mixture, instead of tryptophan (exp. 10). This treatment did not inhibit adsorption.

Experiment 10. Does preincubation with phenylalanine cause inhibition of adsorption in presence of 4 μg per ml L-tryptophan?

B PREINCUBATED WITH	ADSORPTION (METHOD 2)
	%
DL-Phenylalanine 80 μg/ml...............................	70
Control...	53

From the preceding experiments we conclude that the inhibition is created by preincubation with tryptophan, but not by preincubation with phenylalanine. Further, we conclude that the inhibition is established in about 15 minutes, that a few μg of tryptophan are sufficient to establish the inhibition, that the inhibition cannot be overcome by 10 times the amount of tryptophan added to the adsorption tube, and that inhibition established by preincubation with tryptophan is also not overcome by adding phenylalanine in large amounts to the adsorption tube.

Experiment 11 was designed to test whether the inhibition is due to a change in the bacteria or to a change in the medium. A culture of bacteria was inhibited by preincubation with 4 μg per ml L-tryptophan for 60 minutes. It was then mixed with an equal volume of a similar culture incubated without tryptophan. This culture was therefore not inhibited.

Experiment 11. Does preincubation with tryptophan produce a diffusible substance inhibiting the adsorption of T4, 12 on freshly added bacteria?

	ADSORPTION (METHOD 2)
	%
Noninhibited culture......................................	53
Inhibited culture..	3
Mixture...	10

Experiment 11 shows that the medium of the inhibited culture contains a substance or substances, produced by the bacteria in the presence of tryptophan, which prevent the adsorption of phage on bacteria. These substances must be free in solution since adsorption to freshly added bacteria is inhibited as strongly as adsorption to bacteria that caused this metabolic transformation of tryptophan. In the next experiment a similar result was obtained by testing the supernatant of an inhibited culture for its inhibitory power by adding it in equal volume to a noninhibited culture (exp. 12).

Experiment 12.   Test of supernatant of inhibited culture

NONINHIBITED CULTURE MIXED WITH EQUAL VOLUME OF	ADSORPTION (METHOD 2)
	%
Supernatant of inhibited culture.................... ..	2
Basal medium....	25

In experiment 13 known products of tryptophan metabolism were tested individually for inhibitory power. These suspected inhibitory factors were added to a bacterial suspension immediately before adding a mixture of phage, L-tryptophan, and $CaCl_2$. In the adsorption mixtures the concentrations of inhibitory factor, tryptophan, and $CaCl_2$ were 5 μg per ml of each. Ten minutes were allowed for adsorption, and then the mixtures were plated on broth (for the count of total phage) and on plates of F medium (for the count of adsorbed phage), and the supernatant was plated on broth (for the count of free phage).

Experiment 13.   Tests for inhibition of adsorption by known products of tryptophan metabolism

IN ADSORPTION TUBE 5 μg/ml L-TRYPTOPHAN AND 5 μg/ml OF	ADSORPTION	
	METHOD 1	METHOD 2
	%	%
Indole....	3.3	17
Skatole	19	—
Indole-3-acetic acid.......	72	86
Indole-3-propionic acid..	74	74
Tryptamine....	65	72
100 μg/ml DL-serine....	70	62
Control........................ ...	71	78

Of the substances here tested, only indole and skatole showed definite inhibitory power, with indole the more active of the two. All subsequent tests were confined to indole. The inhibition is clearly an inhibition of adsorption. The inhibitory activity of indole on the adsorption of T4,12 in the presence of 5 μg per ml L-tryptophan and $CaCl_2$ is brought out more strikingly in the following titration series (exp. 14).

Experiment 14.   *Titration of indole against 5 μg/ml L-tryptophan*

INDOLE	ADSORPTION (METHOD 2)
μg/ml	%
4	0 5
2	1.7
1	4.4
0 5	13
0.2	32
0.1	39
0	58

Indole can exert a noticeable inhibitory action even at a concentration of 0.1 μg per ml, 50 times lower than that of L-tryptophan.

The preceding experiments have shown that the bacteria produce from L-tryptophan (but not from phenylalanine) a substance or substances capable of inhibiting the adsorption of T4,12 in the presence of L-tryptophan and $CaCl_2$. Among known or suspected products of tryptophan metabolism indole, and to a lesser extent skatole, were found to have such inhibitory activity. Anderson has shown that the function of the cofactor, L-tryptophan, is to activate the phage particles reversibly. It seems natural to assume that the inhibitory substances function by forming competitive combinations with the phage particles.

Several pieces of evidence can be brought forward that may serve to elaborate and in some measure to support this idea. In an experiment already reported (exp. 8.) it was shown that the products of tryptophan metabolism are inhibitory also against phenylalanine. This may indicate that phenylalanine exerts its cofactor activity as a true tryptophan analogue, combining with the phage in the same places where tryptophan combines. Experiment 15 shows that indole is active against every cofactor contained in broth.

Experiment 15.   *Inhibitory action of indole against all cofactors contained in broth*

IN ADSORPTION TUBE 4 mg/ml BROTH AND	ADSORPTION	
	METHOD 1	METHOD 2
	%	%
50 μg/ml indole	18	4.2
Control (no indole)	88	91

The idea that indole acts by establishing a competitive block at the points where tryptophan (or its analogues) reacts with the phage implies that it should inhibit the adsorption of all those, and only those, phage mutants that require tryptophan (or one of its analogues) as cofactors. Specifically, indole should inhibit the adsorption of T4,11, which requires tryptophan but not Ca^{++}, and should not inhibit T4,1, which does not require any cofactor. These inferences are verified by the results of experiments 16 and 17.

Experiment 16.   Inhibition of adsorption of T4,11 by varying amounts of indole

IN ADSORPTION TUBE 5 µg/ml L-TRYPTOPHAN AND INDOLE	ADSORPTION (METHOD 2)
µg/ml	%
40	8.5
20	12
10	18
4	23.5
2	31
1	54
0	75

Experiment 17.   Noninhibition of adsorption of T4,1 by indole

IN ADSORPTION TUBE	ADSORPTION (METHOD 2)
	%
Indole (50 µg/ml)	73
Control	85

Note: Stock T4,1 is not a pure stock. It contains about 10 per cent phage particles giving plaques with fuzzy margins, like the wild type. These 10 per cent are cofactor-requiring. In the experiment just cited these particles were not adsorbed in either the indole or the control tube. These particles, which give clearly distinguishable plaques, were not counted for the evaluation of adsorption.

Experiments 16 and 17 show that indole inhibition is specific for those mutants requiring tryptophan as cofactor for adsorption. It should be noted, however, that T4,11 is about 10 times less sensitive than T4,12 to the inhibitory action of indole.

DISCUSSION

The experiments here reported show that mutations of phages with respect to cofactor requirements do occur and that mutant stocks of reasonable purity can be prepared. Very little is known about the rates at which the mutations occur and about the conditions that influence the rates of mutation. In some cases the rates must be quite high, so high, in fact, that it becomes a matter of chance whether a reasonably pure stock is obtained.

The biochemical deficiencies of the mutants are concerned with the requirements of the phages for the *adsorption* process. It must be clearly understood that the term "adsorption," as it is used in virus research, refers to the occurrence of an irreversible, specific union between virus particles and host. A *physical* adsorption, meaning a static bond, like that between antigen and antibody, may or may not be a step in the biological adsorption process. It seems reasonable to postulate an intermediate step between the hypothetical physical adsorption and the beginning of multiplication of the parasite. For want of a better term we will call this intermediate step the "invasion," meaning a chemical reaction, presumably enzymatically controlled, by which the physically adsorbed particle effects an entry into the host cell. One may imagine that the physical adsorption

process in some cases is reversible and that the observed irreversible adsorption is a combination of physical adsorption followed by invasion. It seems reasonable that biochemical deficiencies of phages (and perhaps of all viruses) should be concerned primarily with the invasion phase of the life cycle of the particles. Once a virus particle has entered a host cell, it is not so likely to be dependent on supplementary factors in the medium, beyond those required by the host itself, since the host cell should provide everything that is necessary.

Anderson's discovery of cofactor requirements of phages, implying that resistance or sensitivity of a host to a virus may be conditioned by the presence or absence of specific factors in the medium, surely constitutes an extraordinary advance in the general approach to problems of virus virulence. Our findings extend this discovery in several directions.

First, they show that mutations occur that make a virus independent of a cofactor. Such a mutation may be looked upon as a host range mutation. Thus, T4,1 will attack strain B in F medium, whereas T4,11 and T4,12 will not. T4,1, therefore, could be looked upon as a host range mutant with respect to the basal medium used.

Secondly they show that viruses with cofactor requirements can be interfered with, apparently by interfering with the utilization of the cofactor by the virus. Thus the attack of T4,11 or of T4,12 on B in the presence of any of the cofactors can be blocked by the presence of indole. In this system indole plays the role of an antivirus drug, and to our knowledge it is the first drug on record that specifically protects a host cell from a virus invasion. This action must not be confused with that of other drugs (2- and 3-pyridyl-alanine, for instance— T. F. Anderson, 1946), which block the *liberation* of active phage particles from infected bacteria, but which do not prevent the adsorption of the phage particles. These drugs, too, effectively block the *spread* of phage infection in a bacterial culture by confining the infection to the cells first attacked.

Third, since the bacteria here studied produce indole from tryptophan, our findings point to a peculiar mechanism of self-protection on the part of the bacteria, converting an activator of an invasive pathogen into an inhibitor. We are inclined to see in this interrelationship a first *rational* link between the metabolic pattern of the host and its resistance pattern. That there do exist links between the metabolic and the resistance pattern is certainly to be expected, and indeed such links have been shown to exist in several instances (E. H. Anderson, 1944, 1946; Wollman, 1947). Some of the bacterial mutations from phage sensitivity to phage resistance have been found to be associated with loss of ability to synthesize either tryptophan or proline. There is in these cases no obvious *rational* connection between the change of the metabolic pattern and the change of the resistance pattern of the bacteria, since the phages involved are not known to have cofactor requirements. The two changes appear to be accidentally linked phenotypic expressions of certain mutational steps. It might be worth while, however, to look more closely into the details of the metabolism of tryptophan and related substances in connection with the resistance pattern.

Finally, the finding that bacteria metabolically produce certain phage inhibi-

tors may help to elucidate a variety of odd findings concerning the development in phage lysate of secondary bacterial growths, which on isolation turn out to be growths of sensitive bacteria. Probably every worker is familiar with this phenomenon, and occasionally one also finds it mentioned in the literature. A particularly striking case has been described recently by Kleczkowska (1945). In none of these cases, apparently, have the "antilysin" or "inhibitor" been identified chemically; and, indeed before Anderson's discovery of the simple chemical nature of a cofactor, it would have seemed a wild surmise to expect the antilysin to be a simple chemical substance. From here on out, the hunt for other simple antilysins is likely to be a lively one.

SUMMARY

Mutant stocks of phage T4 are described that differ from one another with respect to their requirements for cofactors of adsorption. Table 3 summarizes the properties of the principal stocks.

TABLE 3

Properties of mutant stocks of T4

MUTANT STOCK	PLAQUES ON BROTH PLATES	COFACTOR REQUIREMENT	INHIBITION BY INDOLE
T4,1	Sharp	None	No
T4,11	Fuzzy	1 μg/ml L-tryptophan or analogues	Yes 1 μg/ml
T4,12	Fuzzy	Same plus 1 μg/ml Ca^{++}	Yes 0.1 μg/ml

Indole, which is metabolically produced from tryptophan by the bacteria, inhibits the activation of phage by tryptophan.

Possible relations between the metabolic and the resistance pattern of bacteria are discussed.

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THE NUTRITION OF BRUCELLAE: GROWTH IN SIMPLE CHEMICALLY DEFINED MEDIA[1]

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The nutritional requirements of brucellae grown in chemically defined media have been investigated by ZoBell and Meyer (1932a,b), Koser, Breslove, and Dorfman (1941), N. B. McCullough and Dick (1942a,b, 1943), W. G. McCullough et al. (1947), and others. With one exception (McCullough and Dick, 1943), however, the media employed seemed unduly complex by reason of the number of amino acids required in their preparation. Utilization of inorganic ammonium salts as the sole nitrogen source for these organisms has been demonstrated by ZoBell and Meyer (1932a), McCullough and Dick (1943), and Gerhardt and Wilson (1947). Consequently, the present study was undertaken in an effort to obtain a simple, chemically defined medium for cultivation of brucellae.

Interest in this laboratory has centered on the "avirulent" *Brucella abortus* strain 19, widely used for animal immunization. The nutritional requirements of this culture were studied intensively and the findings applied subsequently to a number of other strains.

METHODS

Pyrex glassware was employed for all experimental work; before use it was rigorously cleaned with detergent or acid cleaning solution and rinsed with glass-distilled water. When it was necessary to use chromic acid cleaning solution, extra precautions were taken in rinsing. The highest grade chemicals commercially available were used throughout the study. The accessory growth factors employed were crystalline preparations from reputable sources.

Cultures were grown in 10-ml aliquots of the medium in pyrex milk dilution bottles, which were plugged with cotton enclosed in gauze wrapping. When extended incubation of cultures was necessary, an aluminum foil covering was used to minimize evaporation. The constituents of the medium were made to volume in the culture vessels and sterilized by autoclaving for 20 minutes at 120 C. Duplicate or triplicate cultures were incubated at 35 to 37 C in a humidified atmosphere. Aeration was accomplished by means of a shaker apparatus. Growth usually was evaluated turbidimetrically; light transmittance was determined with an Evelyn photometer, using matched 18-mm test tubes as cuvettes, a 660-mμ filter, and the original, uninoculated medium as the reference. Readings were made on aggregate samples of two or more replicate cultures.

Plate counts were made by the usual poured plate methods. The pH was determined electrometrically.

A majority of the experiments were conducted with *B. abortus* strain 19 (Cotton, Buck, and Smith, 1933), sent to us from the U.S. Department of Agriculture through the courtesy of Dr. A. B. Crawford (U.S.B.A. I., *B. abortus* no. 19-18B, March 8, 1946). This strain is distinguished by its relatively low virulence for experimental animals and by its reaction to dye bacteriostasis (Levine and Wilson, 1947).

Preparation of the inocula for experimental cultures was rigorously standardized, both to minimize variation in numbers and activity as well as to reduce the possibility of nutrient carry-over. Cells were removed carefully from a 24-hour, tryptose agar culture to physiological saline; this suspension was adjusted to a given turbidity and 0.1-ml aliquots of appropriate dilutions were used for the inoculation of experimental cultures. In order that this standard inoculum might be interpreted in terms of numbers of viable cells, a curve was prepared to show the relationship between viable cell count and percentage of light transmittance for suspensions prepared in this manner. Of necessity, early experiments having ammonium sulfate as the nitrogen source were with relatively large inocula; this requirement was subsequently obviated.

RESULTS

The study was begun with attempts to cultivate *B. abortus* strain 19 on the simplified medium of N. B. McCullough and Dick (1943); negative results were obtained in each instance. Similar findings have been reported by Stimmel (1946), Polding (1946), and W. G. McCullough *et al.* (1947). Experiments were then conducted to demonstrate utilization of ammonium salts as the sole nitrogen source by the organism. This accomplished, the study was extended to investigate the nutrition of the organism in relatively simple substrates and to define conditions under which maximum growth might be obtained.

Initial experiments were with a medium arbitrarily devised from the results of N. B. McCullough and Dick (1943) and other investigators. As the study progressed, the medium was changed to incorporate the experimental findings; thus, the various data may not be directly comparable. The basal medium used in most of the work had the following composition: 0.75 per cent sodium chloride, 1.00 per cent dipotassium phosphate, 0.01 per cent sodium thiosulfate, 10.0 μg per ml Mg^{++} (as magnesium sulfate), 0.10 μg per ml Fe^{++} (as ferrous sulfate), 0.10 μg per ml Mn^{++} (as manganous sulfate), 0.20 μg per ml thiamine hydrochloride, 0.20 μg per ml nicotinic acid, 0.04 μg per ml calcium pantothenate, 0.001 μg per ml biotin, and pH adjustment to 6.8 to 7.0.

Limited studies on the respiratory activity of *B. abortus* strain 19 on various energy sources indicated that the rate of oxygen uptake of washed, resting cells in the presence of sodium lactate compared favorably with that for glucose or glycerol.[2] The latter often are employed as energy sources for brucellae. These

[2] Studies on the respiration of brucellae are being extended and will be reported at a future date.

results suggested the incorporation of lactate into the basal medium, which previously had failed to support growth with ammonium sulfate as the nitrogen source and glucose as the carbon source. When the medium was modified by the addition of lactate, moderate growth was obtained. Subsequently, a marked stimulatory effect of glycerol was observed.

The relationships between varying concentrations of lactate and glucose, and of lactate and glycerol, are given in figures 1 and 2, respectively. The results were plotted as three-dimensional graphs to illustrate the relationships between the two independent variables (lactate versus glucose or glycerol concentrations) in terms of the dependent variable (percentage of light transmittance). The
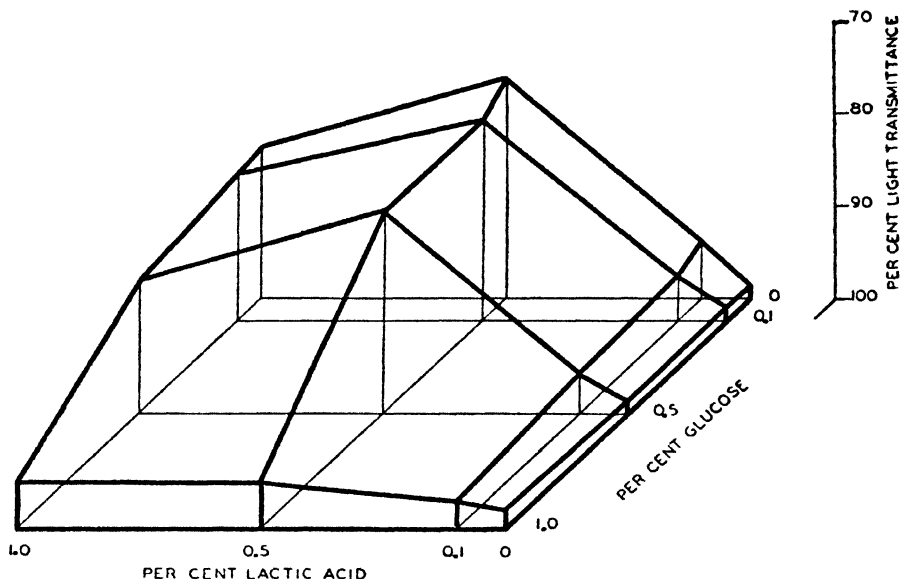
Figure 1 Relationship between varying concentrations of lactic acid and glucose on the growth of *Brucella abortus* strain 19. Medium: Basal medium constituents plus 0.05 per cent (NH₄)₂SO₄. Inoculum: 1 × 10³ viable cells per ml. Incubation: 96 hours, not on shaker

following working conclusions were drawn from the data of figure 1: (1) a concentration of 0.5 per cent lactate was optimal for all concentrations of glucose used; (2) lactate may serve as the sole carbon and energy source; and (3) glucose alone failed to support growth and apparently inhibited growth in the presence of lactate. The following working conclusions were drawn from the data of figure 2: (1) a concentration of 0.5 per cent lactate was optimal for all concentrations of glycerol used; (2) the combined effect of lactate and glycerol together was greater than that of either alone; and (3) although either may serve as the sole carbon and energy source, the effect of lactate was more pronounced than that of glycerol. These results were subsequently extended to determine more accurately the optimal levels of lactate and glycerol, each in the presence of optimal amounts of the other. In this repect, a level of 0.5 per cent lactic acid

was confirmed as optimal, whereas increasing the concentration of glycerol to 3.0 per cent resulted in further increases in the growth response. The range of the optimal concentration of glycerol was found to be very broad.

In this preliminary work, the basal medium included 0.05 per cent ammonium sulfate as the nitrogen source, the culture vessels being incubated statically. That oxygen supply had become a limiting factor soon became evident; consequently, increased aeration was employed in subsequent experiments. Moreover, the use of ammonium sulfate offered serious disadvantages as a nitrogen source: (1) ammonia determinations indicated that appreciable and variable amounts of ammonia (e.g., 12.5 to 36.0 per cent) were lost from the medium on
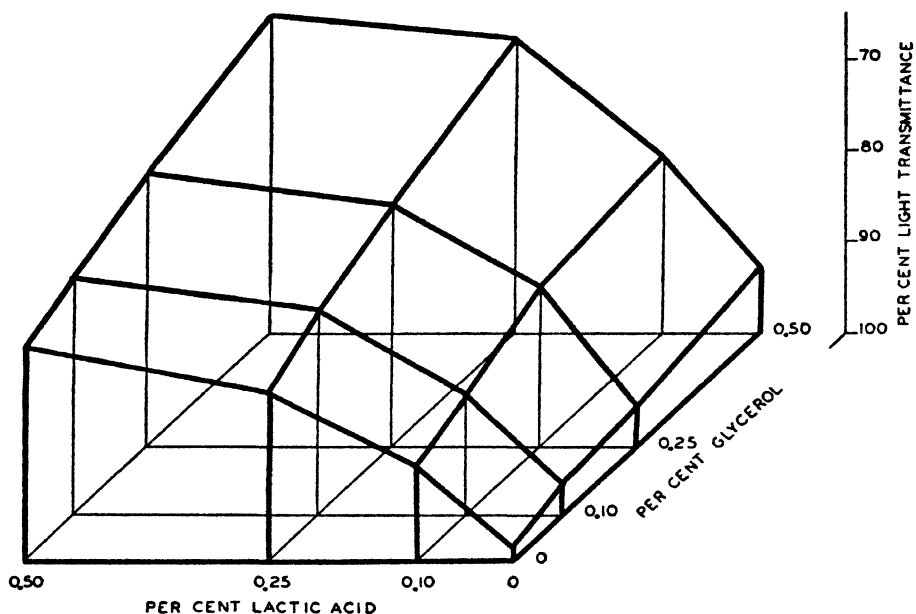
Figure 2. Relationship between varying concentrations of lactic acid and glycerol on the growth of *Brucella abortus* strain 19. Medium: Basal medium constituents plus 0.05 per cent $(NH_4)_2SO_4$. Inoculum: 1×10^5 viable cells per ml. Incubation: 96 hours, not on shaker

autoclaving; and (2) although growth of the organism could be maintained in the medium with prolonged serial transfer, the initiation of growth by small inocula was delayed and sporadic unless the physical character of the medium was altered with small amounts of agar or methyl cellulose. Consequently, a series of experiments was conducted in an effort to obviate these difficulties and yet avoid the use of a multiple nitrogen source. The effect of various nitrogen sources employed singly in the basal medium was investigated. The results indicated that various sources of the inorganic ammonium ion (e.g., ammonium carbonate, hydroxide, phosphate, or citrate) may satisfactorily substitute for ammonium sulfate but with no apparent advantage, either in the growth response or in the chemical properties of the compounds. However, the response of the organism to individual amino acids and related compounds could be demon-

strated.   The favorable results with asparagine, in particular, suggested further
investigation.   Moreover, the stimulatory effects of asparagine on the growth
of brucellae had been observed previously by ZoBell and Meyer (1932a) and
others.

Representative results for the growth response of the organism to asparagine
as the sole nitrogen source are given in table 1.   Not only were the levels of
growth attained comparable to those with ammonium sulfate, but the organism
grew readily from smaller inocula.   From these and subsequent data, the optimal
concentration of DL-asparagine was determined as 0.3 per cent.   Under these
conditions visible growth of *B. abortus* strain 19 became evident after incubation
periods of approximately 1, 3, 5, and 7 days with inocula of 1×10^8, 1×10^6,
1×10^4, and 1×10^2 viable cells per ml, respectively.   The maximum levels of

TABLE 1

The effect of varying concentrations of asparagine on the growth of Brucella abortus strain 19

NITROGEN SOURCE ADDED TO MEDIUM			PERCENTAGE OF LIGHT TRANSMITTANCE	
Percentage of compound	Compound	Nitrogen percentage	1×10^4 cells/ml inoculum, 96 hr	1×10^4 cells/ml inoculum, 232 hr
0	None	0	98.5	100.0
0.05	$(NH_4)_2SO_4$	0.0106	56.0	100.0
0.01	DL-Asparagine	0.0021	83.5	93.0
0.05	DL-Asparagine	0.0106	47.0	52.5
0.10	DL-Asparagine	0.0212	50.5	45.5
0.30	DL-Asparagine	0.0636	49.0	47.0
0 50	DL-Asparagine	0.1060	55.0	100.0
1.00	DL-Asparagine	0.2120	98.5	100.0

Medium: Basal medium constituents plus 0.5 per cent lactic acid and 3.0 per cent glyc-
erol.   Incubation: On shaker.

growth attained in each instance were approximately the same: 45 to 50 per cent
light transmittance and a viable cell count of 1.0 to 2.0 $\times 10^9$ viable cells per ml.
An interesting adjunct to the effect of asparagine is the fact that, at certain
levels of the compound, the culture became distinctly colored, usually brown
ochre but at times a distinct pink.   Centrifugation of such cultures revealed the
pigmentation to be concentrated in the supernatant.

A partial analysis of the utilization of asparagine by the organism is given in
table 2.   Aspartic acid, the amino acid analogue of asparagine, could not be
substituted.   Contrary to expectations, L-glutamine, differing from asparagine
only in the length of its carbon chain, gave no growth response, whereas L-
glutamic acid may serve as the sole nitrogen source.   L-Histidine also may re-
place asparagine.   Of the other amino acids examined, none supported signifi-
cant growth.

As a result of the preceding studies, a simple, chemically defined medium was
formulated, having DL-asparagine as the sole nitrogen source.   Its complete
composition is given in table 3.   *B. abortus* strain 19 was carried through 10
serial transfers in this medium and then examined for possible changes in its
characteristics.   Thus far, no changes have been detected in its cellular mor-

phology, susceptibility to the bacteriostatic effect of thionin blue, or patho-
genicity for guinea pigs. Limited investigation of the dissociation pattern
showed characteristics of 65 per cent rough, 10 per cent intermediate rough, 10
per cent intermediate, and 15 per cent smooth colonies.

TABLE 2

The effect of various amino acids as nitrogen source on the growth of Brucella abortus strain 19

NITROGEN SOURCE ADDED TO MEDIUM			PERCENTAGE OF LIGHT TRANSMITANCE
Percentage of compound	Compound*	Nitrogen percentage	
0.10	DL-Asparagine	0 0212	51.0
0.201	DL-Aspartic acid	0.0212	97.0
0.111	L-Glutamine†	0.0212	100.0
0.222	L-Glutamic acid	0.0212	60.5
0.114	Glycine	0.0212	96.0
0.159	DL-Serine	0.0212	97.0
0.182	L-Crystine‡	0.0212	100.0
0.080	L-Arginine	0.0212	94.5
0.106	L-Histidine	0.0212	74.5
0.274	L-Tyrosine‡	0 0212	100.0
0.250	DL-Phenylalanine	0 0212	99.5
0.155	L-Tryptophan	0.0212	97.5

Medium: Basal medium constituents plus 0.50 per cent lactic acid and 3.0 per cent
glycerol   Inoculum. 1×10^4 viable cells per ml.   Incubation: 240 hours on shaker.
  * With the exception of asparagine and glutamine, these amino acids have been reported
(W. G. McCullough *et al*, 1947) as essential or stimulatory for growth of a strain of *B. suis*.
  † Sterilized separately by filtration and added aseptically to the medium.
  ‡ Present in saturated solution.

TABLE 3

Composition of chemically defined medium

DL-Asparagine	0.30	%
Lactic acid	0.50	%
Glycerol	3.00	%
NaCl	0.75	%
K₂HPO₄	1 00	%
Na₂S₂O₃·5H₂O	0.01	%
Mg⁺⁺ (as MgSO₄·7H₂O)	10.0	μg/ml
Fe⁺⁺ (as FeSO₄·7H₂O)	0.10	μg/ml
Mn⁺⁺ (as MnSO₄·4H₂O)	0.10	μg/ml
Thiamine hydrochloride	0.20	μg/ml
Nicotinic acid	0.20	μg/ml
Calcium pantothenate	0.04	μg/ml
Biotin	0.001	μg/ml

Adjustment to pH 6.8 to 7.0 with NaOH.

The asparagine medium (table 3) was tested for its ability to support growth
of 28 strains of *B. abortus*, *Brucella suis*, and *Brucella melitensis* contained in our
culture collection.   All strains grew readily in an air atmosphere on tryptose
(Difco) agar.   Of these 28 strains, all but 4 grew from an inoculum of approxi-

mately 1×10^4 viable cells per ml within 8 days in the asparagine medium. The yields varied from barely visible growth (97.0 per cent light transmittance) to heavy growth (6.5 per cent light transmittance). Heaviest growth was obtained with *B. abortus* strain ORF; a maximum level of 19.0×10^9 viable cells per ml was reached after 168 hours from an inoculum of approximately 1×10^4 viable cells per ml. With this medium, attempts failed in every instance to cultivate strains of *B. abortus* that required an increased carbon dioxide tension.

DISCUSSION

This investigation has attempted to demonstrate that many strains of *Brucella* may be cultivated on a relatively simple medium, in which all constituents are chemically defined and readily available to most laboratories. The essential nutritive requirements of these organisms may be met by a combination of mineral salts, four accessory growth factors, lactate, glycerol, and a single nitrogen source. Although the inorganic ammonium ion may serve as the sole nitrogen source, best results were attained using DL-asparagine. Glutamic acid or histidine, employed singly, may substitute for asparagine. Whereas McCullough *et al.* (1947) have reported that cystine, tyrosine, phenylalanine, tryptophan, and histidine were essential for growth of a strain of *B. suis* (originally received from Dr. I. F. Huddleson, his strain no. 1772-A), in our hands this strain was among those that grew in the asparagine medium. These results collectively would seem to indicate that combinations of amino acids have a stimulatory rather than an essential role in the nutritional requirements of these organisms.

Although *B. abortus* strain 19 is known to utilize glucose, no response could be obtained with this compound when ammonium sulfate was used as the sole nitrogen source in the basal medium. However, when lactic acid was substituted as the carbon and energy source in an otherwise similar medium, growth was obtained and glucose actually appeared inhibitory. The stimulatory effect of lactate for these organisms has been reported previously, but its preferential use by a strain of *B. abortus* appeared noteworthy. Our data confirm previous observations on the effect of glycerol with the modification that a relatively high level of glycerol was found to be of value, supporting the view that glycerol may function in ways other than as a primary nutrient.

For possible applications to other studies, the medium that supported the best growth of the test organism was investigated for its ability to support the growth of a number of other strains. These included at least five strains of each species of *Brucella*. All of these strains grew in an air atmosphere. Approximately 85 per cent of the cultures initiated growth in the chemically defined medium from relatively small inocula. It seemed probable that those strains which failed to grow might be induced to do so by minor modifications in the medium, as variation in the nutritional requirements of different strains of *Brucella* has been observed previously (e.g., Koser, Bresolve, and Dorfman, 1941). Similarly, it seemed probable that the maximum level of growth attained by any one strain might be considerably improved by minor modifications in the medium. However, cultivation of those strains of *B. abortus* that require an

increased carbon dioxide tension seemingly must await further study of their requirements. It may be noted, in this respect, that N. B. McCullough and Dick (1942b) reported that 41 recently isolated strains of *B. abortus* (requiring an increased carbon dioxide tension) were not successfully grown in an amino acid medium that they employed.

ACKNOWLEDGMENT

The authors thank Mr. H. B. Levine and Dr. D. T. Berman for conducting the dye and virulence tests, respectively. Determinations of virulence and dissociation were also made by the Bureau of Animal Industry, U. S. Department of Agriculture, through the kind co-operation of Dr. C. A. Manthei.

SUMMARY

An investigation of the nutritional requirements of *Brucella abortus* strain 19 resulted in the formulation of a medium consisting of mineral salts, four accessory growth factors, lactate, glycerol, and a single nitrogen source. Although the inorganic ammonium ion may serve as the sole nitrogen source, best results were attained with DL-asparagine. L-Glutamic acid or L-histidine, employed singly, may substitute for asparagine.

Of 28 "aerobic" strains of *Brucella abortus*, *Brucella suis*, and *Brucella melitensis* tested, all but 4 grew in the asparagine medium from relatively small inocula. Yields varied up to 19 billion viable cells per ml.

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STUDIES ON THE ANAEROBIC MICROCOCCI

I. Taxonomic Considerations

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Anaerobic members of the genera *Staphylococcus* and *Micrococcus* have long been known, but, with the exception of the work of Prévot (1933), no serious attempt has ever been made to study these organisms as a group. Our own experience (Foubert and Douglas, 1946) in attempting to identify anaerobic micrococci that had been isolated from plasma and human skin indicated that a further study of the taxonomy of these organisms was needed. This paper presents the results of such a study, together with a determinative key, based primarily on biochemical characteristics, for the identification of these microorganisms.

METHODS AND MATERIALS

Isolation and purification of cultures. Most of the cultures were isolated on medium A, which has the following composition: Difco peptone, 20.0 g; Difco yeast extract, 2.0 g; glucose, 10.0 g; sodium thioglycolate, 1.0 g; methylene blue, 2.0 mg; distilled water, 1,000 ml. The pH is adjusted to 7.3 to 7.5 and drops to 6.9 to 7.1 during sterilization.

The addition of blood or serum to medium A had no effect upon the numbers or types of anaerobic micrococci recovered, nor upon the growth of isolated strains.

The source materials used for the isolation of cultures were skin scrapings, whole tonsils, and material swabbed from the uterus and tonsils of human beings. Suitable dilutions of these materials mentioned above were plated and incubated anaerobically at 37 C for 5 to 7 days. All isolated colonies that developed were stabbed and streaked on deep-butt slants of medium A. Such subcultures were gram-stained after 5 days' incubation and the anaerobic micrococci selected for further purification.

Cultures employed. Table 1 lists the sources and strain designations of the 36 cultures isolated. To these isolates were added strains S1 to S5, recovered from plasma by S. E. A. Gunter of the University of Washington; strain V1 isolated from skin by James Valentine of the University of Washington; strain S6, isolated from plasma by Margaret Pittman of the U. S. Public Health Service; strain BU, isolated from a draining sinus by E. A. Johnson of Baylor University; *Staphylococcus*[2] *aerogenes* BAI, *Staphylococcus anaerobius* RV, *Staphylococcus anaerobius* DUI, *Staphylococcus asaccharolyticus*, and *Staphy-*

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[2] As the genus *Staphylococcus* is now considered invalid (Breed *et al*, 1948), these organisms will be referred to hereafter as *Micrococcus*.

lococcus activus, received from A. R. Prévot of the Pasteur Institute in Paris; and strains U3, U4, and U5, isolated from postpartum uteri by K. Eileen, Hite of the University of Chicago.

Biochemical reactions. The fermentation of various compounds was determined by using fluid medium A without glucose as the basal solution to which the separately sterilized substrates were added in a final concentration of 1 per cent. The utilization of glucose was also determined quantitatively by the analysis of cultures grown in medium A without thioglycolate.

The ability to produce a gassy fermentation of certain constituents (probably amino acids) of peptone and yeast extract was determined by observing shake cultures in solid medium A without glucose. Such fermentations have been commonly referred to as "gas production from peptones," and, although this term is probably a misnomer, it is used here for lack of a more precise substitute.

TABLE 1

Source and strain designation of cultures isolated

SUBJECT	SOURCE	COLONIES FISHED	ANAEROBIC MICROCOCCI FOUND	STRAIN DESIGNATION
M	Skin	53	7	M1 to M7
W	"	98	2	W1, W2
G	"	73	2	G1, G2
A	Vagina	50	3	A1, A2, A3
U	Uterus	60	2	U1, U2
T1	Tonsils	83	5	T1 to T5
T2	"	60	3	T6, T7, T8
T3	"	22	2	T9, T10
T4	"	60	7	T10 to T17
T5	"	24	1	T18
T6	"	27	2	T19, T20

Proteolysis was determined in fluid medium A from which glucose, sodium thioglycolate, and methylene blue had been omitted, and to which was added fresh egg albumen, Loeffler's serum (Difco), 2 per cent skimmed milk, or gelatin. Media for H_2S production and indole production were prepared by substituting proteose peptone no. 3 and Difco tryptone, respectively, for the peptone in medium A. Nitrate and nitrite reduction were tested for in medium A without thioglycolate to which 0.1 per cent KNO_3 or 2 parts per million of KNO_2 had been added. Indole production, nitrate and nitrite destruction, and H_2S production were tested for by the methods recommended by the Committee on Bacteriological Technique (1944).

Coagulase was tested for according to the method of Chapman (1945), and hemolytic activity by the method of Brown (1919), using 5 per cent whole human and rabbit blood.

RESULTS

Detailed descriptions of the organisms are given in a later section under Description of Species. Only those characteristics will be listed below which

are common to all strains or which are pertinent to the taxonomic treatment of the group.

Relation to oxygen: All strains are strictly anaerobic and have shown no tendency to become facultatively anaerobic.

Hemolysin and coagulase: Not produced by any strain.

Catalase: Produced by all strains.

Optimum temperature: Approximately 37 C for all strains.

Pigmentation: Most of the strains are white or grayish white. However, the lactate-fermenting strains exhibit colors varying from tan to yellow brown on solid medium A containing 10 per cent by volume of condensed milk.
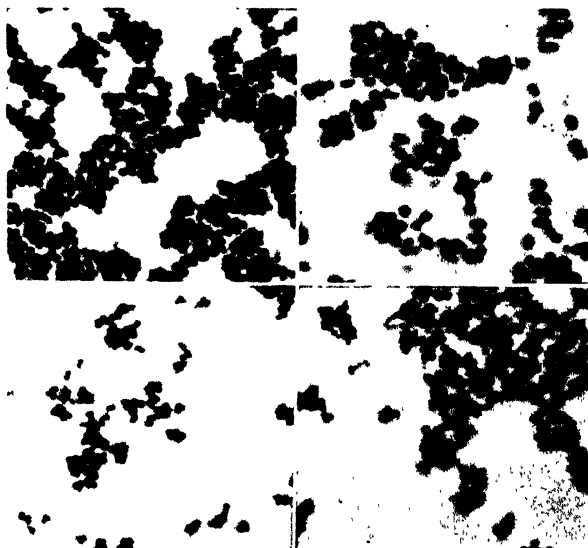
Figure 1. Gram stains of 5-day-old cultures grown in fluid medium A   *Upper left:* strain M1 (*M saccharolyticus   Upper right:* strain U5   *Lower left* strain T3 (*M prevotii*). *Lower right:* strain U3 (*M. variabilis*).   × 1,000.

Gram reaction. All strains are gram-positive, although the lactate-fermenting strains rapidly become gram-negative when grown in medium A in which 1 per cent lactate has been substituted for the glucose. These strains, if not examined during the first 10 to 12 hours of the growth cycle, would be classified as *Veillonella* on the outcome of the gram stain.

Morphology. None of the cultures are encapsulated or motile, and none produce endospores. The majority of the strains are typically mass-forming micrococci of uniform cell size (figure 1, upper left). Cells grouped in pairs and free tetrads can be found in almost every strain, particularly if young cultures are examined. One strain (U5) is characterized by extreme pleomorphism. Many of the cells of this organism are elongated, club-, lemon-, or dumbbell-shaped, and some cells look as if they are budding (figure 1, upper right).

The cell arrangement of certain strains always appeared to consist of small,

irregular masses composed of contiguous tetrads lying in many planes (figure 1, lower left). This arrangement appeared distinctly different from that of typically irregular masses or free tetrads. The grouping of cells into masses of contiguous tetrads, and in some cases contiguous pairs, appeared to a lesser extent in several other strains.

With only a few exceptions, all of the strains were considered to be members of the genus *Micrococcus*. The exceptions were those organisms that consistently formed tetrads; these strains obviously could be placed in the genus *Gaffkya*, and in fact some of them were tentatively classified as such by Foubert and Douglas (1946). However, further consideration has led us to doubt the wisdom of this, for there appears to be too gradual a transition between those strains which form tetrads infrequently and those which form them consistently to enable one to draw any definite line of demarcation. For this reason we prefer to consider the tetrad formers as members of the genus *Micrococcus*. The sole anaerobic member of the genus *Gaffkya* is *G. anaerobia*, which was described by Choukevitch (1911). It seems possible, however, that the organism studied by Choukevitch was an anaerobic sarcina, for this worker's morphological examinations were confined to observations of gram-stained preparations in which packet formation is difficult to detect.

SPECIES DIFFERENTIATION

Species producing gas from peptones. A division of the anaerobic micrococci into two groups can be satisfactorily made on the basis of visible gas formation from peptones. The gases produced in all cases consist of mixtures of CO_2 and H_2, but nothing more is known about the nature of this fermentation. With the exception of strain U5 neither growth nor gas production of the gas formers is enhanced by glucose.

The gas-producing strains may be subdivided on the basis of lactate fermentation. Those strains which ferment lactate are small, mass-forming cocci that are gram-positive in young cultures but, as previously mentioned, readily become gram-negative. All were isolated from tonsils and differ only in the type of growth in broth and in catalase activity. Sugars are not utilized but lactate is fermented with the production of propionate, acetate, CO_2, and hydrogen (Foubert and Douglas, 1948).

The occurrence of gram-negative anaerobic micrococci in saliva was reported by Hall and Howitt (1925), and, although these workers did not report on the fermentation of lactate, their isolates seemed to be very similar to ours except in the gram reaction. Authentic cultures of the organisms of Hall and Howitt are no longer available, but by following the isolation technique of these workers we were able to isolate from saliva what appeared to be gram-negative anaerobic micrococci. However, further examination of such cultures proved them to be lactate fermenters that were gram-positive in young cultures. We consider it highly probable, therefore, that our lactate-fermenting strains are identical with the anaerobic micrococci isolated by Hall and Howitt from saliva.

Hall and Howitt considered these organisms to be identical with *M. gazogenes*

alcalescens anaerobius described by Lewkowicz (1901), but renamed the bacterium *Micrococcus gazogenes*. Because of its apparent failure to retain the gram stain it was later placed in *Veillonella* by Prévot (1933). The fact that the organism is gram-positive, however, necessitates its transfer from *Veillonella* to *Micrococcus*. As pointed out by Bergey *et al.* (1939), the species name *gazogenes* is invalid when applied to a member of the genus *Micrococcus*. Consequently we suggest the species name *lactilyticus*, which reflects the fermentation of lactate carried out by this organism.

Among the non-lactate-fermenting organisms, *Micrococcus niger* appears to be a valid species, which has been adequately described by Hall (1930) and Prévot (1933).

The balance of the non-lactate-fermenters may be subdivided on the basis of gelatin liquefaction. *Micrococcus activus* is the only member of the gelatin-liquefying group and may be recognized not only by its proteolytic activity but also by its active fermentation of several sugars. The results of our examination of one strain received from Prévot are in essential agreement with the description published by Prévot and Taffanel (1945).

In the non-gelatin-liquefying group the highly pleomorphic strain U5 has been separated from the others on the basis of enhanced growth and gas production in the presence of glucose, although the organism uses only a negligible amount of this sugar. Since it is the only strain of its type that we have studied, we feel that it should be left unnamed for the present.

The remainder of the strains in the non-gelatin-liquefying group can be further subdivided on the basis of nitrate reduction and indole formation. Strains A1, A2, U1, and the cultures of *M. aerogenes* and *M. asaccharolyticus* received from Prévot all fall into the group that reduces nitrate and produces indole. None of the cultures use more than a trace of glucose nor do any of them exhibit any proteolytic activity. The only differences we have been able to find among these strains are quantitative, such as the intensity of gas production from peptones and slight differences in cell diameter. All of these cultures agree well with previous descriptions of *M. aerogenes* (Schottmüller, 1912; Prévot, 1933). According to Prévot (1933) *M. asaccharolyticus* differs from *M. aerogenes* in being larger and in failing to ferment sugars or to act upon blood, but these differences were not apparent in the strains of these two organisms available to us.

The non-gelatin-liquefying group that neither produces indole nor reduces nitrate includes six strains (M3, G1, A3, T3, T4, S6) that were isolated from a variety of sources. In these strains cells of two sizes are formed, some of which are twice the diameter of others. The large cells may constitute 20 to 50 per cent of the total cells in 5-day cultures.

This group of six cultures appears to us to be sufficiently homogeneous and different from other described species to justify their inclusion in a new species. We suggest for these organisms the name *Micrococcus prevotii*, in honor of Dr. A. R. Prévot, who has contributed much to our knowledge of anaerobic bacteria.

Species not producing gas from peptones. A primary division of the organisms

in this group can separate those which ferment sugars with the production of acid from those which ferment sugars not at all, or only very slowly and without acid formation. Growth of the organisms in the sugar-fermenting group is strongly enhanced by glucose, whereas growth of the non-sugar-fermenters is not influenced by the presence of this sugar.

In the sugar-fermenting group *M. grigoroffi* appears to be a valid species and has been adequately described by Grigoroff (1905), Prévot (1933), and Prévot and Senez (1944).

Thirteen strains of sugar fermenters, all of which were isolated from skin and blood plasma, are sufficiently different from *M. grigoroffi* to necessitate their inclusion in a new species, and we suggest the name *Micrococcus saccharolyticus* for these strains. These bacteria differ from *M. grigoroffi* in failing to ferment lactose, in not producing indole or H_2S, and in not coagulating milk. There also appear to be clear-cut differences in the nature of the sugar fermentation induced by these two species. Analyses of growing cultures of *M. saccharolyticus* have shown that the principal metabolic products produced in the fermentation of glucose are ethanol, CO_2, formic acid, acetic acid, and traces of lactic acid (Foubert and Douglas, 1946), whereas *M. grigoroffi* produces principally butyric, formic, and lactic acids in glucose broth (Prévot and Senez, 1944).

The strains in the non-sugar-fermenting group grow as well in the absence as in the presence of glucose and possibly cause amino acid fermentation of the type described by Cardon and Barker (1947), in which the only gas produced is CO_2. The organisms in this group may be split along the lines of gelatin liquefaction and morphology. The non-gelatin-liquefying group contains a single species, *M. anaerobius*. The results of our examination of two cultures received from Prévot are in good agreement with the description of *M. anaerobius* published by Prévot (1933).

The gelatin-liquefying group consists of five cultures (U2, U3, U4, T7, and BU). In contrast to the uniform cell size of *M. anaerobius* the cell diameter of each individual strain in this group varies from 0.5 to 1.0 or 1.5 microns, and cells of all sizes may be present in a single culture. We feel that the five strains mentioned above are sufficiently homogeneous and different from *M. anaerobius* to justify their inclusion in a new species, for which we suggest the name *Micrococcus variabilis* because of the characteristic variation in cell size.

DESCRIPTION OF SPECIES

Micrococcus lactilyticus nov. nom.

Cultures examined: 13 strains—T1, T2, T5, T6, T8, T9, T10, T12, T13, T14, T15, T16, and T17 from 4 pairs of normal tonsils; 3 strains—T18, T19, and T20 from 2 pairs of infected tonsils.

Cells spherical; 0.5 to 0.75 microns; occurring singly, in pairs, in tetrads, and in large irregular groups; gram positive in young cultures, but gram-negative thereafter.

Agar colonies: Circular, 0.5 to 1.0 mm, smooth, entire, low convex, opaque, grayish white, and butyrous.

Litmus milk: Complete reduction in 48 hours, no other change.

Utilization of carbon compounds: Carbohydrates are not fermented. Pyruvate, lactate, and malate fermented with the production of abundant gas. Lactate fermented to H_2, CO_2, acetate, and propionate.

Proteolysis: Gelatin, egg albumen, beef serum, and casein not attacked.

H_2S produced; indole not produced; nitrates and nitrites reduced.

Micrococcus aerogenes Schottmüller 1912

Cultures examined: 1 strain received from Prévot; 2 strains, A1 and A2, from the vagina; 1 strain, U1, from the uterus.

Cells spherical; 0.75 to 1.0 micron; occurring singly, in pairs, in tetrads, and in irregular groups some of which appear to be made up of contiguous plates of four arranged in many planes.

Agar colonies: Circular, 0.5 to 2.0 mm, smooth, entire, low convex, opaque, grayish white, and butyrous.

Litmus milk: Completely reduced in 72 hours by moderate gas producers, 75 per cent reduced in 10 days by slight gas producers.

Utilization of carbon compounds: Carbohydrates and lactate not fermented.

Proteolysis: Gelatin, egg albumen, beef serum, and casein not attacked.

H_2S produced; indole produced, nitrate and nitrite reduced.

Micrococcus prevotii n. sp.

Cultures examined: 2 strains, M3 and G1, from the skin; 1 strain, A3, from the vagina; 2 strains, T3 and T4, from tonsils; 1 strain, S6, from plasma.

Cells spherical; some strains 0.6 to 0.8 microns with 20 to 50 per cent of the cells 1.0 to 1.5 microns, some strains 0.75 to 1.0 microns with 20 per cent of the cells 1.5 microns; occurring singly, in pairs, in tetrads, and in irregular groups, some of which appear to be made up of contiguous plates of four arranged in many planes.

Agar colonies: Circular, 0.5 to 1.0 mm, smooth, entire, low convex, some strains translucent, some strains opaque, gray to grayish white, butyrous.

Litmus milk: Complete reduction in 3 to 4 days, no other change.

Utilization of carbon compounds: Some strains produce slight acid from glucose, fructose, galactose, mannose, maltose, and raffinose, but not from lactose, starch, inulin, salicin, glycerol, or mannitol. Other strains produce no acid detectable by pH changes from any of these compounds. Lactate and malate are not fermented.

Proteolysis: Gelatin, egg albumen, beef serum, and casein not attacked.

None or slight H_2S production; indole not produced; nitrates not reduced.

Micrococcus saccharolyticus n. sp.

Cultures examined: 5 strains, S1, S2, S3, S4, and S5, from 3 bottles of plasma; 8 strains, M1, M2, M4, M5, M7, G2, W1, and V1, from skin.

Cells spherical; 0.6 to 0.8 microns for some strains, 0.8 to 1.0 microns for other strains; occurring singly, in pairs, in tetrads, and in irregular groups.

Agar colonies: Circular, 0.5 to 1.0 mm, smooth, entire, convex, opaque, white, and butyrous.

Litmus milk: Slight reduction; no other change.

Utilization of carbon compounds: Acid but no visible gas produced from glucose, fructose, mannose, glycerol, and sucrose. No acid from arabinose, galactose, maltose, lactose, raffinose, starch, inulin, salicin, mannitol. Lactate and malate not fermented. Glucose fermented to CO_2, ethanol, acetic acid, formic acid, and traces of lactic acid.

Proteolysis: Gelatin, egg albumen, beef serum, and casein not attacked.

H_2S and indole not produced; nitrates and nitrites reduced.

Micrococcus variabilis n. sp.

Cultures examined: 1 strain, U2, from the uterus during pelvic inflammatory disease; 2 strains, U3 and U4, from 2 postpartum uteri; 1 strain, T7, from a normal tonsil; 1 strain, BU, from a draining sinus.

Cells spherical; 0.5 to 1.5 microns (all intermediate sizes may be present in a single culture); occurring singly, in pairs, in tetrads, and in irregular groups which appear to be made up of contiguous pairs arranged in many planes.

Agar colonies: Circular, 0.5 to 1.0 mm, smooth, entire, low convex, opaque, grayish white, butyrous.

Litmus milk: Slight reduction; no other change.

Utilization of carbon compounds: No acid produced from carbohydrates. Lactate not fermented.

Proteolysis: Gelatin liquefied; egg albumen, beef serum, and casein not attacked.

Slight H_2S production; indole not produced; nitrates not reduced.

Strain U5

Cultures examined: 1 strain, U5, from the uterus.

Cells spherical, 0.5 to 2.5 microns in diameter; pleomorphic, evidencing rod, lemon, club, dumbbell, and budding forms.

Agar colonies: Circular, 0.5 to 1.0 mm, entire, low convex, opaque, grayish white, butyrous.

Litmus milk: Complete reduction in 24 hours; no further change.

Utilization of carbon compounds: Slight acid formed from glucose, mannose, sucrose, and lactose. No acid from arabinose, raffinose, starch, salicin, glycerol, or mannitol. Lactate not fermented.

Proteolysis: Gelatin, egg albumen, beef serum, and casein not attacked.

H_2S produced; indole not produced; nitrate not reduced.

KEY TO THE SPECIES OF THE ANAEROBIC MICROCOCCI

A. *Produce visible gas from peptones*
   1. Ferment lactate..............................*M. lactilyticus* nom. nov.
   2. Do not ferment lactate
      a. Colonies in deeps pigmented black by 6 days..............*M. niger*

      b. Colonies not so pigmented
         1) Liquefy gelatin....................................*M. activus*
         2) Do not liquefy gelatin
            a) Growth and gas production enhanced by glucose......Strain U5
            b) Growth and gas production not enhanced by glucose
               (1) Indole produced; KNO_3 reduced; gas production slight to
                   abundant...............*M. asaccharolyticus, M. aerogenes*
               (2) Indole not produced; KNO_3 not reduced;
                   gas production slight...................*M. prevotti* n. sp.
B. *Do not produce visible gas from peptones*
    1. Ferment glucose
      a. Ferment lactose.......................................*M. grigoroffi*
      b. Do not ferment lactose.....................*M. saccharolyticus* n.sp.
    2. None or only a slight fermentation of glucose
      a. Do not liquefy gelatin; cell size uniform...............*M. anaerobius*
      b. Liquefy gelatin; cell size variable................*M. variabilis* n. sp.

SUMMARY

A comparative study has been made of 52 strains of anaerobic micrococci, 36 of which were freshly isolated from the skin, vagina, uterus, and tonsils, while the remainder, including all of the available accepted species of anaerobic micrococci, were obtained from other workers. It was concluded that *M. niger M. activus, M. aerogenes, M. grigoroffi*, and *M. anaerobius* are valid species. The one strain of *M. asaccharolyticus* available to us could not be distinguished from *M. aerogenes*.

Three new species, *M. prevotti*, *M. saccharolyticus*, and *M. variabilis* were described, and one species, *Veillonella gazogenes*, was redescribed and transferred to the genus *Micrococcus* as *M. lactilyticus*.

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STUDIES ON THE ANAEROBIC MICROCOCCI

II. The Fermentation of Lactate by Micrococcus lactilyticus

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Micrococcus lactilyticus is a strictly anaerobic microorganism that is indigenous to the mouth of man and possibly other animals. The metabolism of this organism is interesting in that it fails to ferment sugars but carries out a vigorous fermentation of pyruvate, malate, and lactate (Foubert and Douglas, 1948). Lactate fermentation in the absence of an additional hydrogen acceptor is rare among bacteria but has been found to occur in *Propionibacterium* (van Niel, 1928) and *Butyribacterium* (Barker and Haas, 1944).

This paper presents the results of quantitative experiments to determine the nature of the lactate fermentation conducted by *M. lactilyticus*.

METHODS

The results reported here were obtained with one strain (T5) of *M. lactilyticus*. The fermentations were conducted at 37 C in an all-glass fermentation vessel of the type designed by Professor H. A. Barker of the University of California. The medium used contained 0.2 per cent Difco yeast extract and 1 per cent sodium lactate. Under these conditions the fermentations were complete in 24 hours, and the bacteria utilized 3.5 to 4.0 mM of lactate per 100 ml of medium.

The fermentation gases were collected over mercury and analyzed volumetrically. Lactate was determined by the method of Barker and Summerson (1941). Volatile acids were determined quantitatively by the double distillation method of Friedemann (1938). The identity of the volatile acids was tentatively determined by the method of Osburn, Wood, and Werkman (1936) and verified by partition chromatography. The chromatographic method used was essentially a combination of the procedures of Elsden (1946) and Ramsey and Patterson (1945). The individual acids, after resolution on silica gel, were chromatographed with known acids to establish their identity.

RESULTS

The only fermentation products produced in detectable amounts from lactate by *M. lactilyticus* are propionic and acetic acids, carbon dioxide, and hydrogen. Table 1 summarizes the results of a typical fermentation. The fermentation can be seen to be fairly similar to that carried out by the propionic acid bacteria, but it differs in that hydrogen gas is produced. The ratio of propionate to acetate is considerably less than 2.0, the value required by the equation for the propionic acid fermentation of lactate:

$$3 \text{ lactate} \rightarrow 2 \text{ propionate} + \text{acetate} + CO_2.$$

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This is to be expected, however, for on theoretical grounds the formation of each mole of hydrogen gas should spare the reduction of 1 mole of lactate to propionate. The data in Table 1 bear this out; the ratio, propionate + hydrogen to acetate, is 1.99.

TABLE 1

Fermentation of lactate by Micrococcus lactilyticus (T5)

(mM per 100 mM lactate fermented)

Propionic acid.....................................		63.7
Acetic acid.......................................		39.5
Carbon dioxide...................................		39.1
Hydrogen..		14.2
Carbon recovery (%).............................	103.6	
O/R index.......................................	0.99	

As cell suspensions were shown to have no action upon formate, the fermentation can be postulated to occur according to the following over-all reactions:

$$CH_3 \cdot CHOH \cdot COOH \rightarrow CH_3 \cdot COOH + CO_2 + 4H \quad (1)$$

$$2H \rightarrow H_2 \quad (2)$$

$$CH_3 \cdot CHOH \cdot COOH + 2H \rightarrow CH_3 \cdot CH_2 \cdot COOH \quad (3)$$

Hydrogen formation probably occurs by a diversion to hydrogen gas (equation 2) of part of the available hydrogen produced in the oxidative phase of the fermentation (equation 1), whereas propionate can be considered to arise from the reduction of lactate (equation 3).

SUMMARY

Micrococcus lactilyticus carries out a propionic acid fermentation of lactate, which differs from the classical propionic acid fermentation in that hydrogen gas is produced.

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THE MICROBACTERIA

I. Morphological and Physiological Characteristics

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The genus *Microbacterium* was proposed by Orla-Jensen in 1919. Four species were described, viz., *Microbacterium lacticum*, *Microbacterium flavum*, *Microbacterium liquefaciens*, and *Microbacterium mesentericum*. The organisms of this genus presented a physiological gradient with the saccharolytic acid former (*M. lacticum*) at one extreme and the proteolytic gelatin liquefier (*M. liquefaciens*) at the other extreme. Later (1921), with reference to the taxonomic position of this genus, Orla-Jensen stated: "*Microbacterium* is to be understood as merely a provisional collective name for gram-positive rods of size a little smaller than ordinary bacteria. In biological respects some of these rods (*Bacillus acidophilus*) are closely related to the true lactic acid bacteria, whereas others approach the *Tetracocci*[1] or the aerobic bacilli." No further detailed investigations were made on this genus for 14 years.

Robertson (1927) encountered *M. lacticum* in his study of thermoduric organisms isolated from heated milk. His data agreed well with those given by Orla-Jensen (1919) for *M. lacticum*. An interesting conclusion of Robertson was the statement that *M. lacticum* and *Lactobacillus thermophilus* were identical. However, the description of *L. thermophilus* by Ayers and Johnson (1924) leaves little doubt that these organisms are distinctly different.

A valuable contribution to our knowledge of the genus was made by Wittern (1933). Her work confirmed and extended that of Orla-Jensen (1919). A major portion of her investigation dealt with the taxonomic position of *M. mesentericum*, which she allocated to the genus *Mycobacterium*. Jensen (1932) made a morphological study of the genus. His observations led him to the conclusion that *M. mesentericum* was a proactinomyces and hence should be *Proactinomyces mesentericus*. Other recommendations by Jensen were that *M. lacticum* and *M. liquefaciens* be designated as *Corynebacterium lacticum* and *Corynebacterium liquefaciens*. Further studies (1934) led him to the conclusion that *M. flavum* was actually a *Mycobacterium* and therefore should be designated as *Mycobacterium flavum*.

Hansen (1938) found that hydrocyanic acid or iodoacetate inhibited the respiration of *M. lacticum* and indicated that the microbacteria differed from the true lactic acid bacteria not only in catalase content but also in their hemin content. Speck (1943) took issue with the conclusions of Jensen (1932, 1934), stating that to place the *Microbacterium* in the genera *Mycobacterium* or *Coryne-*

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[1] *Tetracoccus* as used by Orla-Jensen refers to acid-producing forms of *Micrococcus* and *Sarcina*. Much of the acid they produce is acetic acid, and many liquefy gelatin. The colonies are generally yellow, orange, or pink.

bacterium on a morphological basis was not justified. He concluded that any system of classification should indicate a close relationship between the genera *Microbacterium*, *Propionibacterium*, and *Lactobacillus*.

Orla-Jensen (1943), in an attempt to show the taxonomic relationships of the microbacteria to other groups, was prompted to diagram their very close relationship to the corynebacteria and actinomycetes. The Danish master, however, was well aware of the implications involved when he concluded, "*Der Vollstandigkeit halber habe ich—mehr oder weniger berechtigeweise · in unserem System auch die* Mikrobakterien *und die* Propionibakterien *angebracht. Ob es richtig ist,* Corynebakterien *und* Actinomyceten *von den* Mikrobakterien *und von* Bm. bifidum *entspringen zu lassen muss die Zukunft lehren.*"

The fifth edition of *Bergey's Manual* (1939) places *Microbacterium* in the second genus of the family *Bacteriaceae*. The four species recognized by Orla-Jensen (1919) are included in the genus. In the sixth edition of this manual (1948), the genus has been transferred to the family *Lactobacteriaceae* and is the second genus of the tribe *Lactobacilleae*. *M. liquefaciens* has been placed in an appendix to this genus and *M. mesentericum* is classified as *Nocardia mesenterica*.

It is apparent that conflicting opinions exist both as to the characterization and the systematic position of these organisms. This investigation presents data that may be of some significance in evaluating their taxonomic position.

EXPERIMENTAL METHODS AND RESULTS

Cultures. For the purposes of this investigation, known cultures were received from the sources indicated in table 1. In addition, original isolations were made using the following procedure: Five-ml samples of raw or pasteurized milk were pipetted into sterile test tubes and heated at 61 C for 30 minutes or 73 C for 16 seconds. The samples were cooled immediately in an ice bath preceding the preparation of decimal dilutions. Originally several media were employed for the isolation of the microbacteria, but, during the course of the investigation, it was found that a medium of the following composition was at least equivalent and sometimes superior to the others:

Peptone	0.5 g
Meat extract	0.3 g
Fructose	0.1 g
K_2HPO_4	0.4 g
KH_2PO_4	0.1 g
Distilled water	100.0 ml

Adjust to pH 7.0 before sterilization

The inoculated plates were incubated at 30 C for 3 days. Microscopic preparations were made from colonies measuring a few mm or less in diameter. If short gram-positive, nonsporing rods were present, the colony was transferred to litmus milk and incubated at 30 C for 3 days, after which time a subculture was made to an agar slant. After incubation the agar slant culture was checked to ascertain purity and then placed under refrigeration for future study. Forty

cultures were isolated in this manner. Of these, five representatives were included in this study.

Unfortunately, cultures of *M. liquefaciens* were not obtained either by the isolation technique described above or from the sources listed in table 1.

Cultural characteristics. Observations on the colonial morphology of the microbacteria used in this study were made on streak plate cultures grown on Difco tryptone glucose extract agar with skim milk added and incubated 3 days at 30 C.

TABLE 1

Source and designation of cultures

CULTURE DESIGNATION	CODE NUMBER	SOURCE OF CULTURES
M. lacticum	3	Prof. S Orla-Jensen,
" "	6	Biochemical-Technical Institute,
M. flavum	8	Copenhagen, Denmark
" "	9	
" *lacticum*	8180	American Type Culture Collection,
" "	8181	Georgetown University,
		Washington, D. C.
" *lacticum*	513	Dr. Johanna Westerdijk,
" "	516	Centraalbureau voor Schimmelcultures,
" *flavum*	531	Baarn, Netherlands
" "	534	
" *lacticum*	S-2	Dr. M. L. Speck,
" "	30-3	National Dairy Research Laboratories, Inc.,
" *flavum*	342-S1	Baltimore, Maryland
" *lacticum*	1PM3	Original isolations,
" "	3RM2	University of Maryland,
" "	3PMb-9	College Park, Maryland
Microbacterium sp.	3RM5	
" "	3RMb-1	

The surface colonies of *M. lacticum* were 0.25 to 0.50 mm in diameter. They were punctiform, glistening, smooth, convex, pearl white or gray, translucent, and finely amorphous. They may be described as "dewdrop" colonies. Cultures OJ3, OJ6, S-2, 30-3, 513, 516, 8180, 1PM3, 3RM2, and 3PMb-9 were of this type (figure 1A). Cultures 342-S1 and 3RM5 were also of this type but were cream or slightly yellow in color.

The surface colonies of *M. flavum* averaged 2.0 mm in diameter. They were glistening, smooth, convex, and cream or canary yellow. They were also generally round and possessed an even edge. Cultures 531, 534, OJ8, and OJ9 were of this type. The pigment varied from the color of rich cream (OJ8) to a very bright canary yellow (534) (figure 1B).
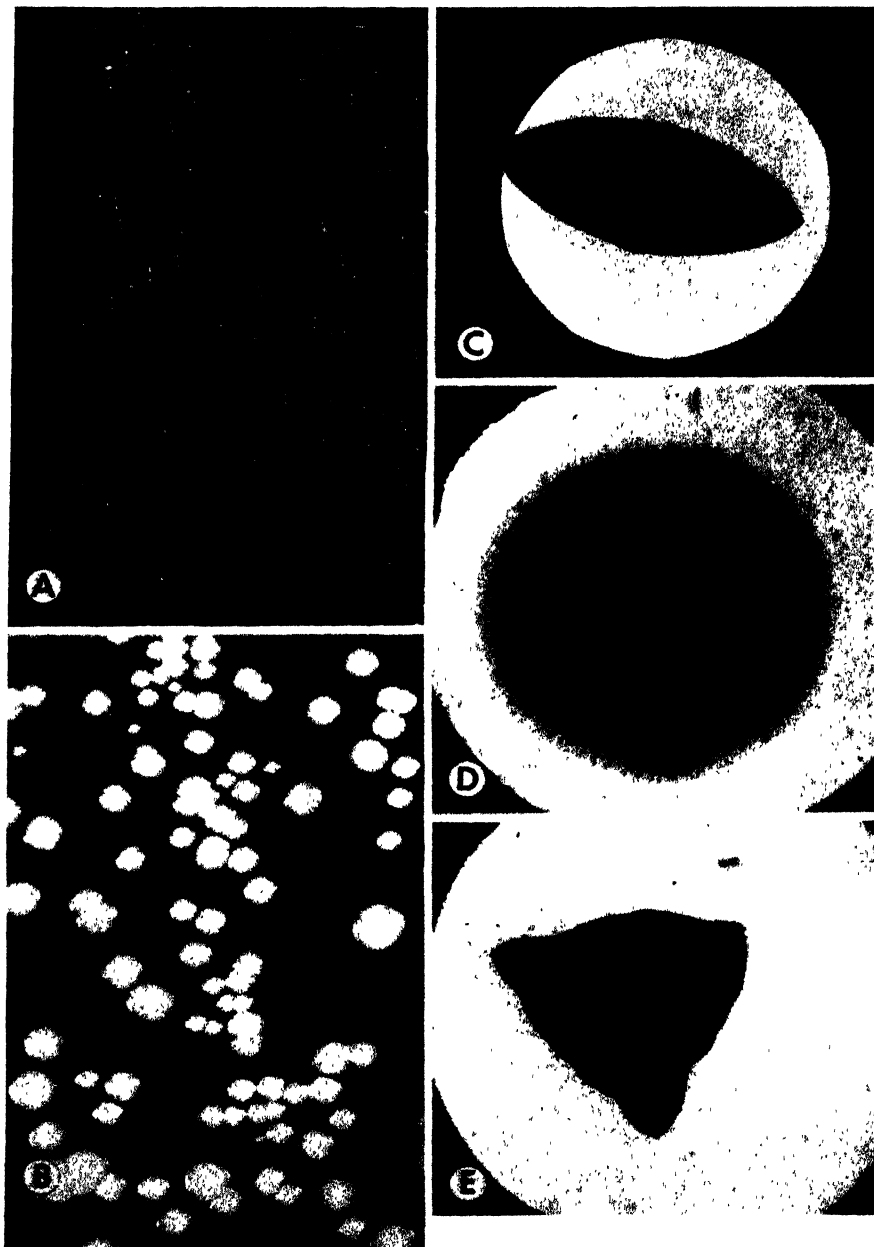
Figure 1. A, surface colonies of *M. lacticum* S-2 (×5½). B, surface colonies of *M. flavum* OJ8 (×5½). C, D, E, subsurface colonies of *M. lacticum* 3RM2 (×57).

The chief difference between the colonies of *M. lacticum* and *M. flavum* appeared to be one of color. Generally the colony diameter of *M. lacticum* averaged

0.5 mm, whereas that of *M. flavum* averaged 2.0 mm.   This was not considered a distinguishing characteristic.   Subsurface colonies were usually lens-shaped, although round, "lumpy," and diffuse colonies were occasionally encountered. All of these subsurface colony types could occur from the same culture (figures 1C, D, E).   Growth under anaerobic conditions was much slower, requiring 5 to 7 days' incubation at 30 C for colony formation, and pigmentation was minimal.
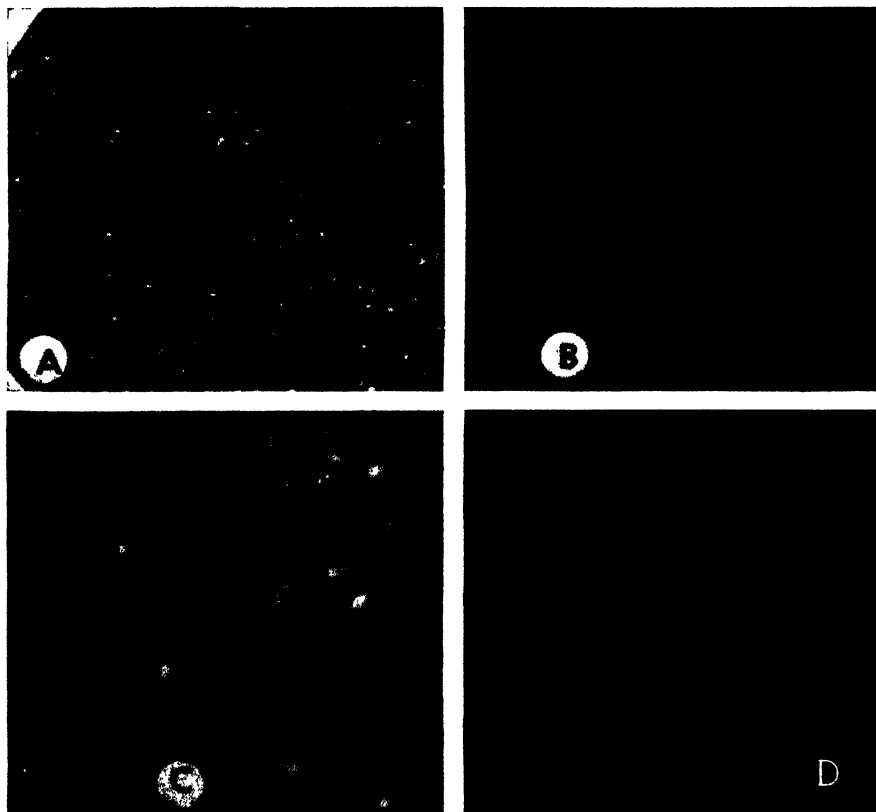
Figure 2.   Morphological types of *Microbacterium*.   A, type 1 (*M. lacticum* 3RM2).   **B,** type 2 (*Microbacterium* sp. 3RM5).   C, type 3 (*M. flavum* OJ8).   D, type 4 (*M. lacticum* 513).

Yeast extract proteose peptone glucose broth cultures of the organisms generally showed varying degrees of turbidity from faint to heavy.   Most of the cultures formed a stringy, viscid, sediment after 4 days at 30 C.   Agar slant cultures showed good surface growth after 2 days at 30 C.   In the case of *M. lacticum*, the growth was greenish white, white, or gray.   Many cultures showed a translucent film of growth over the surface of the slant.   *M. flavum* gave rather heavy growth, which was usually cream or canary yellow.

Morphological characteristics.   The morphology of the cultures used in this

study agreed with the descriptions of previous investigators. All of the cultures were found to be gram-positive (Hucker's modification), nonmotile (hanging drop), and nonsporeforming. They showed bipolar granulation when stained with Loeffler's methylene blue and were non-acid-fast when stained with Ziehl-Neelson's carbol fuchsin and decolorized with acid alcohol. On the basis of cell morphology, the cultures have been arbitrarily divided into four types as follows:

Figure 3. Morphological type 1 of microbacteria as shown by the electron microscope (× ca. 26,000). *M. lacticum* strain 3RM2 shadowed with chromium.

Type 1. Short, thin rods (0.5 to 0.7 by 1.4 to 2.1 μ), occasionally club-shaped, were regarded as the classical type morphology of *M. lacticum*. Angular and palisade arrangements were frequently noted in preparations made from cultures grown on solid media. Granulation was observed when the cells were stained with Loeffler's methylene blue and was pronounced in cultures grown in milk and stained with Newman-Lampert stain. Representatives of this type are shown in figures 2A and 3. The electron micrograph (figure 3) reveals that the cell

contents are unusually dense. Cultures 3RM2, 1PM3, S-2, 30-3, 3PMb-9, 8180, and 8181 are of this type. Organisms that are probably representative of this type have been noted by Orla-Jensen (1919), Robertson (1927), Wittern (1933), Düll (1933), and Speck (1943).

Type 2. In this type were coccobacillary and wedge-shaped rods as described by Orla-Jensen (1919) and Jensen (1934). The organisms resembled micrococci.
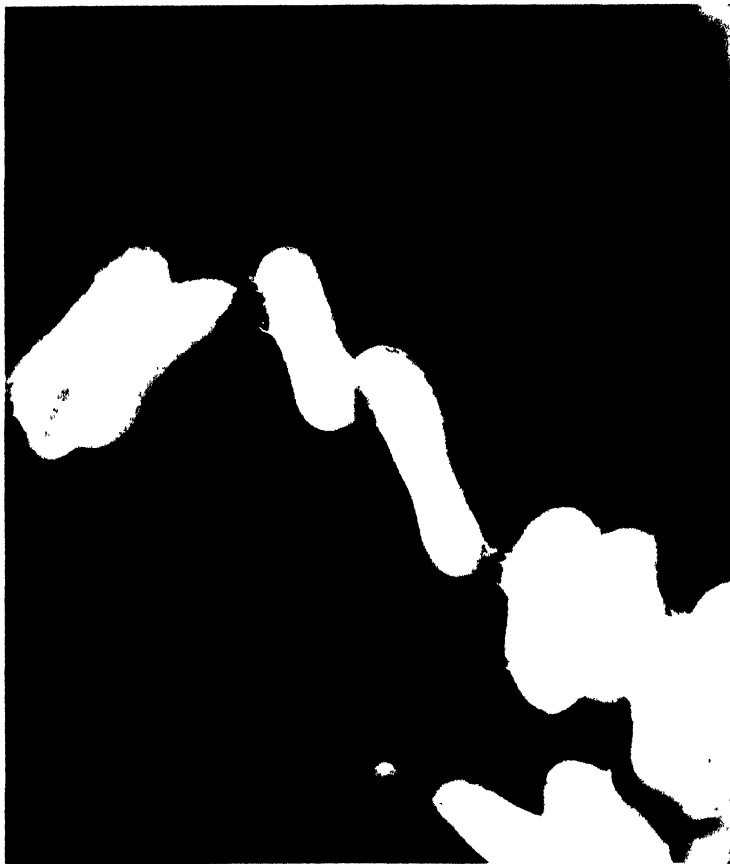
Figure 4. Morphological type 3 of microbacteria as shown by the electron micrograph (× ca. 26,000). *M. flavum* strain OJ8 shadowed with chromium.

In most instances the major axis of the cell was only slightly longer than the minor axis. They had diameters ranging from 0.7 to 1.4 μ. Granulation was apparent in preparations made from milk cultures. Cultures 3RMb-1, 516, 534, 342-S1, 3RM5, and OJ6 were of this type (figure 2B).

Type 3. *M. flavum* was representative of this type. The cells measured 0.7 to 0.9 by 1.4 to 2.6 μ, and none were observed that were 10 μ long, a characteristic attributed to them by Orla-Jensen (1919) and Wittern (1933). Cultures

OJ8, OJ9, and 531 were representatives of this type (figures 2C, 4). Here again the electron micrograph reveals the extreme density of the protoplasm.

Type 4. This type had long, thin, filamentous cells. This type has been described by Wittern (1933) as representative of *M. mesentericum.* Culture 513 (labeled *M. lacticum*) was of this type. It had an unusually long, threadlike
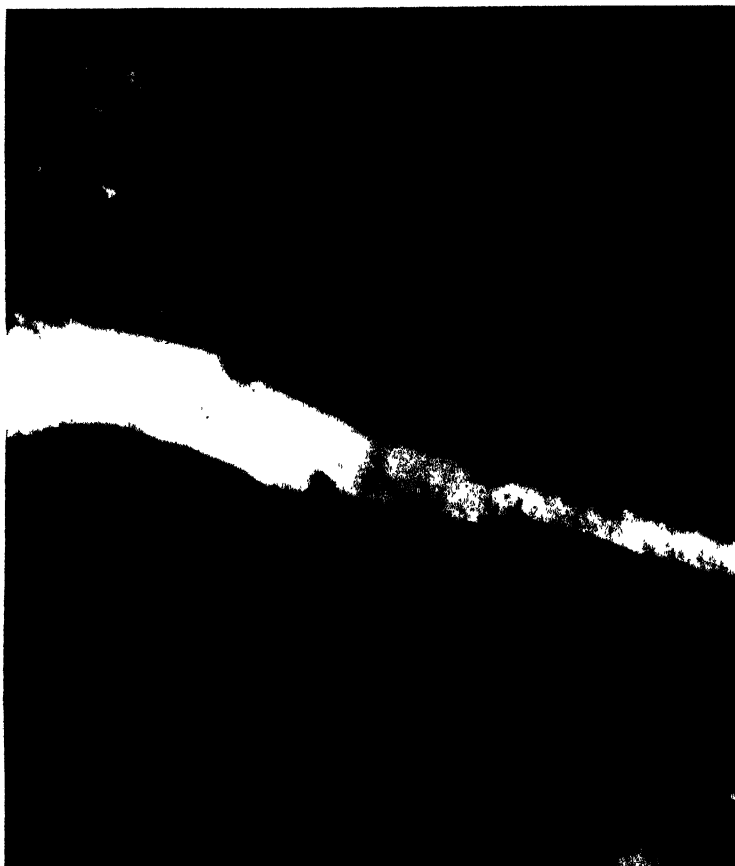
Figure 5. Morphological type 4 of microbacteria as shown by the electron microscope (× 26,000). *M. lacticum* strain 513 shadowed with chromium.

appearance (30 μ long) and differed markedly from all cultures used in this study (figures 2D, 5).

When microbacteria were grown under anaerobic conditions, their morphology was not significantly altered. The rod-shaped appearance of some cultures was more difficult to detect and, in general, all were more coccuslike in appearance.

Physiological characteristics. Forty physiological reactions were determined for each of the cultures, the results of which are presented in table 2. The media and methods used conformed as closely as possible to those described in leaflet

V of the *Manual of Methods for Pure Culture Study of Bacteria* (1944). Determinations of carbohydrate, alcohol, and glucoside fermentations were performed using a cystine agar base[2] to which was added 1 per cent of the test substrate previously sterilized by filtration. Cultures were incubated at 30 C, and the results were read at the end of 4 and 8 days' incubation unless otherwise indicated. Acid production in skim milk was determined after 7 days' incubation at

TABLE 2
Physiological reactions of the genus Microbacterium

CULTURES		Arabinose	Xylose	Galactose	Cellobiose	Lactose	Maltose	Melibiose	Sucrose	Trehalose	Melezitose	Raffinose	Dextrin	Glycogen	Starch	Glycerol	Dulcitol	Mannitol	Esculin	Salicin	MR-VP	85C-2½ min	Nitrite	Final pH glucose phosphate broth	Hippurate hydrolysis	10% NaCl	% Acid skim milk	Litmus milk
M. lacticum	OJ3	0	0	A	A	A	A	0	0	A	0	0	A	0	A	0	0	A	0	0	+0	0	+	5.30	0	0	0.24K	A
" "	OJ6	0	0	A	A	A	A	0	A	A	0	0	A	0	A	0	0	A	0	0	+0	0	+	5.35	0	0	0.07	R
" "	516	0	0	A	0	0	A	0	A	A	0	0	A	0	A	0	0	A	0	0	+0	0	+	5.10	0	0	0.29K	sl A
" "	8180	0	0	A	0	0	A	0	0	A	0	0	A	0	A	0	0	A	0	0	+0	+	+	5.78	0	0	0.28K	A
" "	30-3	0	0	A	A	A	A	0	0	0	0	0	A	0	A	0	0	A	0	0	+0	+	0	5.60	0	0	0.19	A
" "	8-3	0	0	A	A	A	A	0	A	A	A	0	A	0	A	0	0	A	0	A	+0	0	0	5.50	0	0	0.29K	A
" "	1PM3	0	0	A	A	A	A	0	A	0	0	0	A	A	A	0	0	0	0	A	00	+	0	6.10	0	0	0.31K	A
" "	3RM2	0	0	A	A	A	A	0	A	A	A	0	A	A	A	0	0	A	0	0	00	0	+	6.00	0	0	0.37K	A
" "	3PMb-9	0	0	A	A	A	A	0	A	A	A	0	A	0	A	0	0	A	A	A	+0	0	0	5.58	0	0	0.23K	A
" "	513	A	A	A	A	0	A	0	A	A	0	0	A	A	A	0	0	0	0	0	+0	0	+	5.15	0	0	0.09	AR
" "	8181	0	0	A	0	A	0	0	0	0	0	0	0	0	0	0	0	A	0	0	+0	0	0	5.57	0	0	0.04	0
M. flavum	OJ8	0	0	A	0	0	0	0	0	0	0	0	0	0	0	0	0	A	0	0	00	0	0	6.85	0	+	0	R
" "	OJ9	0	0	0	0	0	0	0	0	A	0	0	0	0	0	0	0	A	0	0	00	0	0	6.70	0	+	0.07	0
" "	531	0	0	A	0	0	0	0	0	0	0	0	0	0	0	0	0	A	0	0	00	0	0	6.65	0	+	0.06	0
" "	534	0	A	A	A	A	A	A	A	A	0	A	A	0	A	A	0	A	0	0	00	0	0	7.00	0	0	0	0
" "	342-S1	A	A	A	A	A	A	0	A	A	0	A	A	A	0	A	A	0	A	+0	0	+	5.80	0	0	0.17	0
Microbacterium sp.	3RM5	A	A	A	A	A	0	A	A	A	0	A	A	0	0	A	0	A	A	A	00	0	0	7.08	0	+	0.09	A
"	3RMb-1	A	A	A	A	A	0	A	A	A	0	A	A	0	0	A	0	A	0	A	00	0	0	7.00	0	0	0.14	A

0 = No change or no reaction.
A = Acid.
+ = Positive reaction or growth.
K = Curdled.
R = Reduction.
* All the cultures studied had the following characteristics: glucose, fructose, and mannose were fermented; rhamnose, inulin, adonitol, and sorbitol were not fermented; catalase was produced; gelatin was not liquefied; and indole and hydrogen sulfide were not produced.

30 C. Twenty ml of skim milk culture were titrated with 0.1 N sodium hydroxide to the phenolphthalein end point and calculated as lactic acid. The heat resistance of the organisms was determined by subjecting a freshly inoculated vial containing 5 ml of buffered glucose yeast extract broth (pH 6.6) to a temperature

[2] Cystine 0.5 g, trypticase 20.0 g, agar 3.5 g, sodium chloride 5.0 g, sodium sulfite 0.5 g, phenol red 0.017 g, H₂O 1,000 ml, pH 7.3. Provided by the Baltimore Biological Laboratory, Inc., in dehydrated form.

of 85 C for $2\frac{1}{2}$ minutes. Immediately after this treatment, the vials were cooled in an ice bath and subsequently incubated at 30 C for 7 days to determine viability.

The organisms in the genus *Microbacterium* employed in this study had the following physiological characteristics in common: glucose, galactose, fructose, and mannose were fermented; catalase was produced; rhamnose, inulin, adonitol, and sorbitol were not fermented; gelatin was not liquefied and indole and hydrogen sulfide were not produced.

Because of the fermentation of arabinose, xylose, raffinose, and glycerol it was evident that some of the organisms differed from the typical reactions of either *M. lacticum* or *M. flavum* by fermenting all of these substrates. This group, which attacked the widest range of carbohydrates, has been tentatively designated as *Microbacterium* sp. Table 3 presents a grouping of the cultures on the basis of arabinose, xylose, raffinose, and glycerol fermentation. Two cultures received from other sources designated as *M. flavum* (cultures 534 and 342-

TABLE 3

Differential biochemical reactions of M. lacticum, M. flavum, and Microbacterium sp.

SPECIES OF MICROBACTERIUM	ARABINOSE	XYLOSE	RAFFINOSE	GLYCEROL
M. lacticum (9 cultures)............	0	0	0	0
M. flavum (3 cultures).....	0	0	0	0
Microbacterium sp.				
534	0	A	A	A
342-S1.............	A	A	A	A
3RM5	A	A	A	A
3RMb-1........	A	A	A	A

0 = No change.
A = Acid.

S1), as well as two original isolations, have been placed with the group designated as *Microbacterium* sp. Significantly, all four of these cultures are of morphological type 2. The group designated as *Microbacterium* sp. attacks the widest range of carbohydrates with the production of acid. *M. lacticum* is comparatively less active in this respect, whereas *M. flavum* attacks the least number of carbohydrates. None of the microbacteria produced gas from carbohydrates detectable by the usual cultural techniques.

The microbacteria are weakly proteolytic, if at all, since they neither liquefied gelatin nor digested the casein of unneutralized skim milk. Hydrogen sulfide and indole were not produced. Reduction of nitrate by *M. lacticum* was variable, but none of the typical *M. flavum* strains were able to effect this change. It is apparent that this characteristic is of limited value in identifying species of *Microbacterium*.

Although *M. lacticum* was reported to be able to survive 85 C for $2\frac{1}{2}$ minutes in milk, only 3 of the cultures used in this study were able to survive this treatment in yeast extract proteose peptone broth (pH 6.6). Undoubtedly, the milk

itself enhances the thermal resistance of the organisms. Although valuable for isolation purposes, thermal resistance does not appear to be a satisfactory characteristic for classification of the microbacteria. Determinations of this type present many difficulties that have been emphasized by Rahn (1945).

Although Orla-Jensen (1919) reported that *M. flavum* grew as a flaky precipitate in 10 per cent salt broth, this characteristic was not observed. Growth in the salt broth appeared as a viscous sediment similar to that which appeared in the salt-free broth.

SUMMARY AND KEY

From the foregoing data the following characterization of the microbacteria is presented:

(1) *Microbacterium* sp. Gram-positive, nonsporing, nonmotile, wedge-shaped or coccobacillary rods (0.7 to 1.4 μ diameter). Non-acid-fast.

Form small surface colonies about 1 mm in diameter. Colonies smooth, round, convex, glistening, white or gray. Internal structure amorphous.

Catalase-positive.

Acid in litmus milk, no coagulation.

Gelatin not liquefied.

Indole not formed.

Hydrogen sulfide not formed.

Acid from arabinose, xylose, glucose, galactose, fructose, mannose, cellobiose, lactose, melibiose, sucrose, trehalose, raffinose, and glycerol.

Aerobic, facultatively anaerobic.

Optimum temperature 30 C.

(2) *Microbacterium lacticum*. Gram-positive, nonsporing, nonmotile, short rods (0.5 to 0.7 by 1.4 to 2.1 μ). Non-acid-fast. Granulated when stained with methylene blue. Characteristic angular and side-by-side arrangements in preparations made from solid media. At times appear coccobacillary or wedge-shaped.

Colonies same as *Microbacterium* sp.

Catalase-positive.

Acid in litmus milk; coagulation variable.

Gelatin not liquefied.

Indole not formed.

Hydrogen sulfide not formed.

Acid from glucose, galactose, fructose, mannose, maltose, dextrin, and usually starch. Fail to ferment arabinose, rhamnose, xylose, melibiose, raffinose, and glycerol.

Aerobic, facultatively anaerobic.

Optimum temperature 30 C.

(3) *Microbacterium flavum*. Gram-positive, nonsporing, nonmotile, short rods (0.7 to 0.9 by 1.4 to 2.6 μ). Non-acid-fast. Granulated when stained with methylene blue. May appear coccobacillary or wedge-shaped.

Form small surface colonies about 2.5 mm in diameter. Colonies smooth, round, convex, glistening, cream or canary yellow.

Catalase-positive.

Litmus milk unchanged.

Gelatin not liquefied.

Indole not formed.

Hydrogen sulfide not formed.

Acid from glucose, fructose, mannose, and mannitol. No acid from arabinose, rhamnose, xylose, raffinose, dextrin, starch, or glycerol.

Aerobic, facultatively anaerobic.

Optimum temperature 30 C.

The following key has been constructed that may aid in the identification of the microbacteria.

    I. Acid from glycerol and raffinose
        A. Acid from arabinose and xylose
            1. *Microbacterium* sp.
    II. No acid from glycerol and raffinose.
        A. Acid from maltose and starch
            2. *Microbacterium lacticum*
        B. No acid from maltose and starch
            3. *Microbacterium flavum*

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THE ROLE OF PEROXIDE IN THE BIOLOGICAL EFFECTS OF IRRADIATED BROTH

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Evans (1947) has reported that treatment of sea water with X-radiation deleteriously affects the survival and activity of *Arbacia* eggs and sperm. This effect can be duplicated by H_2O_2 and can be completely negated by the addition of catalase. Such biological effects of irradiations have been studied in a number of laboratories (Barron *et al.*, 1947; Giese, 1947). Stone *et al.* (1947, 1948) increased the mutation rate of *Staphylococcus aureus* to penicillin and streptomycin resistance by ultraviolet irradiation of the substrate prior to inoculation of the organisms. They suggest that the mutant individuals arise as a result of the assimilation of modified substrate molecules. A similar enhancement of the mutation rate results when the organisms are grown in a nutrient broth that has been treated with hydrogen peroxide prior to inoculation (Wyss, Stone, and Clark, 1947). Since very sensitive chemical methods failed to show any residual hydrogen peroxide at the time of inoculation of the treated broth, the mechanism appears analogous to the irradiation experiments.

The similarity of the effects produced on proteins and peptones by H_2O_2 and radiations has been reviewed by Arnow (1937). Fernau (1923) concluded that the results of a treatment with Roentgen rays, ultraviolet, and alpha particles on albumin solutions were identical with those produced by peroxide. Lieben (1927) states that the disappearance of the color reaction for tyrosine and tryptophan in proteinaceous solutions exposed to ultraviolet can be duplicated with H_2O_2. After the solutions stand for several days, the color reactions reappear. This process can be hastened by the addition of spongy platinum to decompose traces of peroxide in the solutions. However, Taylor, Greenstein, and Hollaender (1948) showed that the changes in viscosity of sodium thymus nucleate solutions that occurred following X-radiation could not be duplicated by H_2O_2 at 10^{-2} to 10^{-5} M.

It is generally agreed (Duggar, 1936; Heyroth, 1941) that the major portion of the action resulting from the direct treatment of organisms with radiation is not due to peroxide. Yet, Thoday and Read (1947) have demonstrated that the X-radiation of *Vicia faba* in the absence of oxygen resulted in a significantly lowered incidence of chromosomal aberrations. Crabtree and Cramer (1934) report the inhibition of tumor cells by radiation to be dependent on oxygen. Even the complete absence of oxygen does not preclude peroxide formation in an irradiated aqueous medium.

The nature of the substrate that is subjected to irradiation will determine the

type of reaction. Baumgartner (1936) notes that the irradiation of carbohy-drates results in a marked production of acids, approximately half of which is formic. Bacterial growth is inhibited but occurs if sufficient base is added to the medium to neutralize the acid. Stevens (1936) reviews the literature on oil ir-radiation and reports experiments proving that peroxides are the only bacter-icidal substances in emulsions of irradiated cod-liver oil. Treatment of mucoid strains of pneumococci and Friedlander's bacilli with sublethal doses of the ir-radiated oil results in dissociation to intermediate types. Stevens points out that the peroxides adhered to the organisms and that their antibacterial action continued even after the organisms were removed and resuspended in solutions of dilute reducing substances. Photographic plates were fogged by exposure to the irradiated oil, indicating either secondary radiations or a volatile oxidizing substance. Blank and Arnold (1935) found that irradiation of nutrient agar with ultraviolet light produced a nonvolatile, thermostable, toxic substance that was capable of diffusing through the medium.

In our work on the induction of mutations by irradiation of the substrate with ultraviolet we have investigated the inhibitions resulting in irradiated medium and the relationship with the mutation phenomenon.

EXPERIMENTAL RESULTS

Sterile nutrient broth was exposed in a large petri dish so that the depth did not exceed 3 millimeters at a distance of 10 cm from the arc of a Hanovia SC 2537 lamp operating at 100 milliamperes. After a suitable exposure time the broth was transferred aseptically to a sterile bottle and inoculated immediately from a log phase culture of *S. aureus* FDA strain no. 209. Plate counts were made in nutrient agar to which penicillin and streptomycin were added when indicated. The catalase was a special sample that had no preservative added and was obtained from the Vita Zyme Laboratories, Chicago, Illinois. It was sterilized by filtration and contained about 1 per cent active protein. Concen-trations of catalase expressed in the results are percentages of this solution.

When the young culture of *S. aureus* was inoculated at the rate of 100,000 cells per ml into irradiated broth, growth occurred during the first 2 hours. After this, in the broth irradiated 10 minutes under the conditions detailed above, cell multiplications ceased and the number of cells remained constant for several hours, but eventually growth resumed. In broth irradiated for 20 minutes, initial growth occurred, but then multiplication stopped and the population re-mained constant for an extended period or declined slightly. In broth irradiated for 30 minutes, again an initial growth occurred that ceased at 2 hours, and the population then decreased rapidly. Such population curves differ markedly from those obtained by the action of most disinfectants. In fact, this initial multiplication is very similar to that which occurs when cells are irradiated di-rectly. Lea (1947, p. 325) observes that a bacterium "killed" by irradiation will divide once or even twice after being sown on a nutrient medium but will not give rise to a colony. There is the difference that although the organisms do not continue to divide promptly in our irradiated medium, they do form col-onies when removed, diluted, and plated on nutrient agar.

The size of the inoculum is an important factor in these experiments as is shown on figure 2.   In broth irradiated for 30 minutes, an inoculum of 10^7 cells per ml grows as well as the control.   With inocula of one-tenth that many cells, growth is temporarily arrested between the second and fourth hours, whereas 10^5 cells per ml die off rapidly after a slight initial growth.   The experiment repeated with the inoculum washed twice by centrifuging with saline gave essentially identical results.   This suggested an intracellular enzyme, and the addition of 0.05 per cent of the sterile catalase solution to the broth before inoculation completely removed the inhibitory principle even in broth that had been irradiated for 30 minutes.   The normal growth curve could be obtained in such catalase-treated broth even with small inocula.
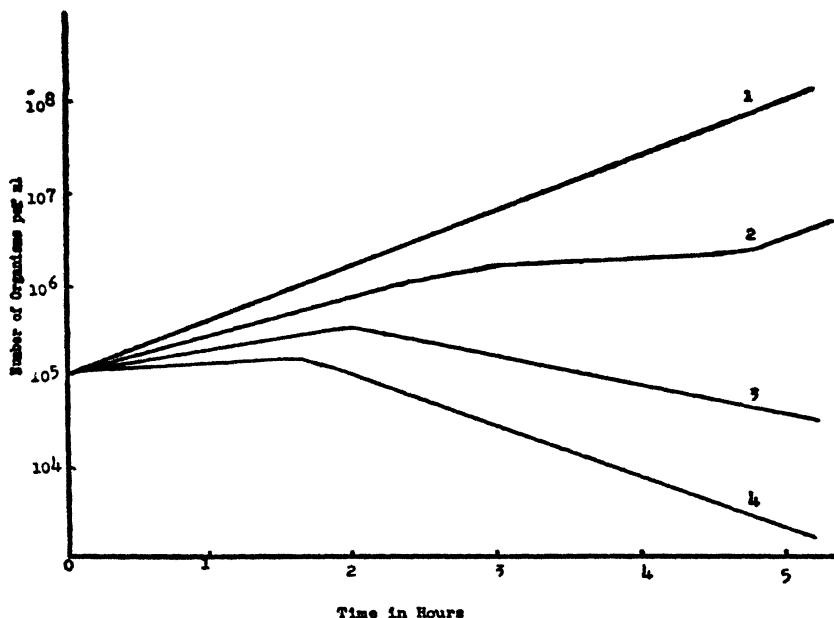
Figure 1.   Growth of *S. aureus* in irradiated broth.   1. Controls.   2. Irradiated for 10 minutes.   3. Irradiated 20 minutes.   4. Irradiated 30 minutes.

The amount of hydrogen peroxide present in the irradiated broth cannot be measured by the $TiCl_3$ method (Wyss, Stone, and Clark, 1947).   Therefore, an attempt was made to estimate the amount of peroxide produced by our lamps. Considerable variation occurs not only among various lamps, even of the same make and model, but also with the age of the lamp.   Measurement of the increase in peroxide in irradiated distilled water revealed that about 1.4 ppm H_2O_2 was obtained as a maximum.   Obviously, at such a concentration the rate of photodecomposition of the peroxide is equal to its formation.   But if one extrapolates the initial rate of peroxide formation (the dotted line in figure 3) and assumes that in nutrient broth the peroxide reacts with the broth components as soon as it is formed, one has a crude measure of the dosage of the broth with

peroxide formed by the different times of irradiation. Various doses of H_2O_2 were added to broth, and the biological action of such broth was compared with broth irradiated for the time indicated by figure 3 as producing an equivalent peroxide concentration. The results indicated that the behavior of such equivalent broths on both inhibition of growth and the production of mutations was similar.

This suggests that the catalase is decomposing not only hydrogen peroxide but also some organic peroxide or other oxidized molecule present in the broth. Confirmation of this was obtained by measuring the oxygen output of irradiated broth when mixed with catalase. Five ml of strongly irradiated broth were placed in a Warburg respirometer and 0.05 ml of the catalase solution were
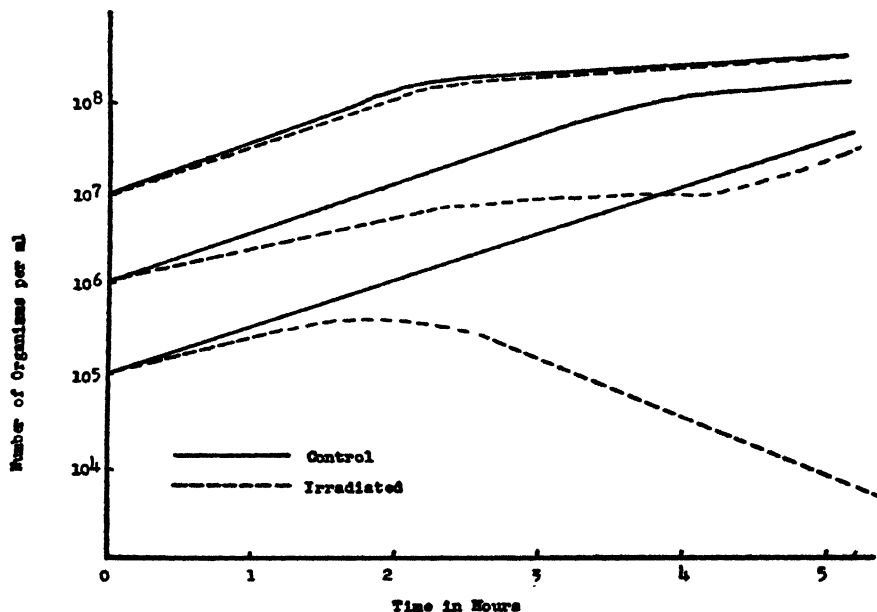
Figure 2. Effect of inoculum size on growth of *S. aureus* in irradiated broth

placed in the side arm. After equilibration the enzyme was mixed with the broth and the evolution of gas was measured. The controls included irradiated broth with heat-inactivated catalase and without catalase, and nonirradiated broth with catalase. Another control was included with KOH in the center cup to rule out the production of CO_2 from the irradiated broth by a decarboxylase contaminant of the catalase. Actually, in broth irradiated for an hour we found an O_2 output equivalent to 18 ppm H_2O_2. The oxygen evolution was complete within a few minutes after the catalase was dumped from the side arm. A typical experiment is recorded in table 1.

The effect of inoculum size on the mutation rate to penicillin and streptomycin resistance is recorded in table 2. It will be observed that in irradiated broth with an inoculum size of 1 million cells per ml a marked enhancement of the
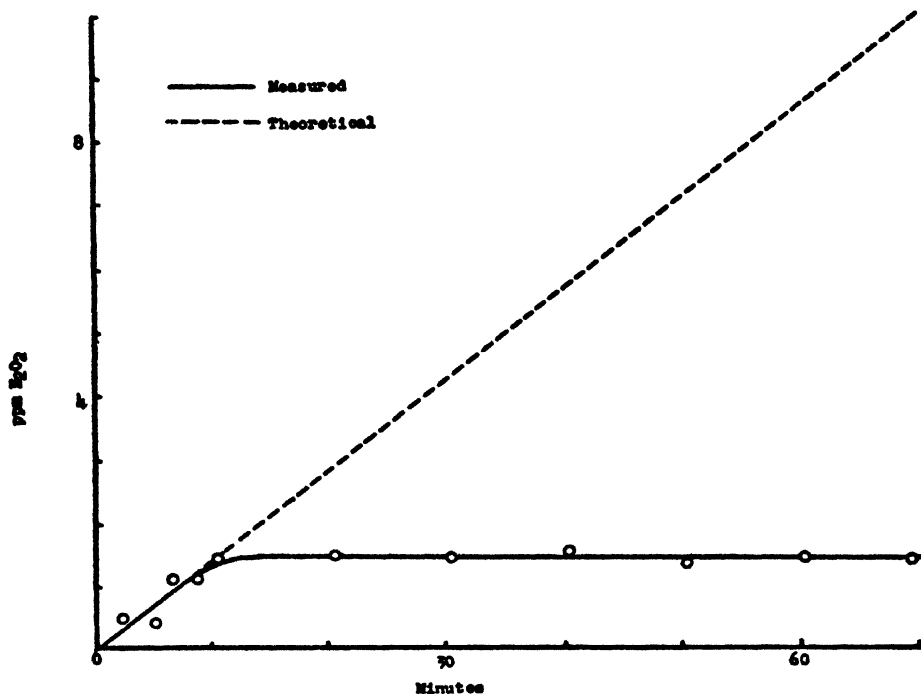
Figure 3. Peroxide formation in irradiated distilled water

TABLE 1

O_2 output from irradiated broth upon the addition of catalase as measured in the Warburg respirometer

	μl O_2	ppm H_2O_2
Unirradiated broth...............................	0	0
Unirradiated broth + catalase....................	1	0.6
Irradiated broth.................................	2	1.2
Irradiated broth + catalase......................	28	16.8
Irradiated broth + boiled catalase...............	3	1.8
Irradiated broth + catalase + KOH in center cup.	30	18.0

Five ml of broth per flask + 0.05 ml catalase in the side arm where indicated.

TABLE 2

Effect of inoculum size on mutation rate in irradiated broth expressed as mutants per million

INOCULUM	PENICILLIN MUTANTS		STREPTOMYCIN MUTANTS	
	Irradiated	Controls	Irradiated	Control
ml				
10^7	8	6	35	25
10^6	24	3	1,435	63
10^5	34	4	2,100	59

mutation rate is observed and that with smaller inocula this rate is increased somewhat. However, when larger inocula are used, the mutation rate drops to that of the control cultures. The experiment repeated with washed inocula gave identical results. When catalase is added to the broth before or after the irradiation, the mutation rate to penicillin and streptomycin resistance of cultures grown in such broth remained essentially that of the control cultures.

If H_2O_2 is an important cause of mutation, it should be possible to enhance the natural mutation rate of *S. aureus* by adding to the medium a poison for the enzyme catalase. Azide, which is a general poison for iron-porphyrin type enzymes, will not only inhibit catalase, the enzyme-destroying peroxide, but will also inhibit the cytochrome system, thus forcing more of the aerobic respiration through the peroxide-producing spontaneous oxidation of the flavoprotein enzymes. Experiments in which the staphylococci were grown in subinhibitory concentrations of sodium azide showed a marked increase in the rate of mutation to penicillin-resistant and streptomycin-resistant forms.

The mutating effect of H_2O_2 on washed cells of *S. aureus* was tested in a series of experiments. When 10 to 1,000 ppm of H_2O_2 were allowed to kill a large percentage of the washed cells suspended in saline and the survivors were assayed for mutants, it was found that the mutation rate did not differ from that of the control population. If the washed cells of which a large fraction were killed by H_2O_2 were inoculated into nutrient broth and allowed to grow, the resultant culture showed no enhancement of the mutation rate.

SUMMARY AND CONCLUSIONS

There is a marked similarity between certain biological effects produced by ultraviolet irradiation of nutrient broth and by the addition of hydrogen peroxide to the broth. The effects of both can be negated by catalase. Because the direct action of peroxide on the cells in the absence of an organic substrate failed to increase the mutation rate it seems reasonable that the results are due to the direct or indirect interference of an organic peroxide in some metabolic function. Whether the catalase reacts with the organic peroxide (or other oxidized molecule) or only with the hydrogen peroxide in equilibrium with it, is not known. In either case, since the cells studied contain active catalase, it is necessary to postulate that some reaction must occur such as the union of the labile substance with an anabolic enzyme, which protects the mutating molecule from the catalase of the cell.

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THE EFFECT OF HUMAN SERUM ON THE DILUTION BIOASSAY OF PENICILLINS G, X, AND K

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It was shown by Bigger in 1944 that, contrary to earlier reports, serum inactivated penicillin and influenced markedly the results obtained in its bioassay. It was subsequently found (Eagle, 1947a) that K was far more susceptible in this regard than the other molecular species of penicillin. It was further shown that penicillin was bound by serum protein (Chow and McKee, 1945), and that penicillin K was bound to a greater degree than X, G, or dihydro F (Tompsett, Schultz, and McDermott, 1947).

Both the inactivation of penicillin and its binding varied with the serum concentration. In order to eliminate or at least to minimize this complicating factor in the bioassay of penicillin by a dilution method, Tompsett and his coworkers added normal, pooled human serum so as to equalize its concentration in all the assay tubes. It is the purpose of this communication to present a table of corrective factors that compensate for the inhibitory effect of various concentrations of human serum on the assay of penicillins G, X, and K in the modified Kirby-Rantz method previously described (Eagle, 1947b). With the application of these factors it becomes unnecessary to complicate the assay by the introduction of added normal serum.

METHODS AND MATERIALS

Eleven individual human sera were tested with respect to their effects on the bioassay of penicillins G and K. Four other individual sera were separately tested with penicillin X; these results paralleled closely four additional determinations with a specimen pooled from approximately 25 patients.

In the control assay containing no serum, varying amounts of a penicillin solution in broth (0.8, 0.72, 0.6, 0.48, 0.4 ml, etc.) were adjusted to a total volume of 0.8 ml with broth. A 4 per cent broth suspension of human group O erythrocytes was inoculated with the C-203 strain of *Streptococcus pyogenes*. This was then added to each tube in the amount of 0.5 ml to serve as the hemolytic indicator. The end point was the tube containing the smallest amount of penicillin that had completely prevented hemolysis after 6 hours' incubation at 37 C followed by 10 hours at room temperature.

In order to determine the effect of serum on the results of this assay, dilutions of penicillin in 96, 48, 24, 12, and 6 per cent human serum were similarly distributed, and the volume was adjusted to 0.8 ml with the corresponding penicillin-free serum dilution. For example, one volume of 1:1,000,000 penicillin in

broth was diluted with 24 volumes of 50 per cent serum to give a 1:25,000,000 dilution in 48 per cent serum. This was distributed and the volumes adjusted to 0.8 ml by the addition of 48 per cent serum in broth. The amount of penicillin necessary to inhibit hemolysis in the presence of varying concentrations of serum compared to the amount necessary in its absence provided a direct measure of the degree to which the penicillin activity had been inhibited by serum.

TABLE 1

The effect of human serum on the bioassay of penicillins G, X, and K

(For technique of assay, see text)

PENICILLIN SPECIES	CONCENTRATION OF SERUM (%) IN PENICILLIN ASSAY*		RELATIVE ACTIVITY† OF PENICILLIN SERUM NUMBER											RELATIVE ACTIVITY		CORRECTIVE FACTOR‡	
	In serum dilution	After addition of red blood cell suspension	1	2	3	4	5	6	7	8	9	10	11	Mean	Median	Mean	Median
G	96	59	25	—	38	—	33	28	42	38	38	45	25	34.7	38	2.9	2.6
	48	29.5	50	[90]	50	56	50	42	50	45	60	45	38	51.8	50	1.9	2.0
	24	14.8	55	83	90	67	83	67	67	75	90	60	60	72.5	67	1.4	1.5
	12	7.5	—	100	—	110	110	83	83	90	—	100	100	97	100	1.0	1.0
X	96	59	40	50	40	—	46	46	47	50				45.6	46	2.2	2.2
	48	29.5	60	60	60	—	60	60	60	63				60	60	1.7	1.7
	24	14.8	80	80	80	80	75	75	75	85				78.8	80	1.3	1.3
	12	7.5	90	100	100	100	90	90	90	85				93	90	1.1	1.1
	6	3.8	100	100	90	100	100	100	98	85				96.7	100	1.0	1.0
K	96	59	3.5	—	8	—	5	4	6.6	6	—	6	5	5.5	5.5	18.2	18.2
	48	29.5	8.4	20	15	17	13	10	13	12.5	15	12.5	10	13.5	13	7.4	7.7
	24	14.8	19	40	33	30	30	30	15	40	15	30	25	27.9	30	3.6	3.3
	12	7.5	38	67	50	60	60	33	40	50	50	50	50	50	50	2.0	2.0
	6	3.8	56	100	60	100	80	60	60	67	80	67	60	71.8	67	1.4	1.5

* Smallest amount of serum which, brought up to a total volume of 0.8 ml and incubated with 0.5 ml of a human red blood cell suspension, completely inhibited hemolysis by the C-203 strain of *Streptococcus pyogenes*.

† For example, 0.1 ml of serum in total of 0.8 ml = 12.5 per cent; after the addition of 0.5 ml of red blood cell suspension used as the hemolytic indicator, the serum concentration becomes 8.3 per cent.

‡ That is, the factor by which the apparent penicillin content of a given serum dilution must be multiplied in order to correct for the inhibitory effect of the serum on the assay.

RESULTS

All the experiments with penicillins G, X, and K in human serum are summarized in table 1. The median inhibitory effect of varying concentrations of human serum on the activity of these penicillins is shown graphically in figure 1. As had been previously shown, it was apparent that the assay of penicillin K was affected to a far greater degree than that of the other species of penicillin. As little as 6 per cent serum had a significant effect on the bioassay of penicillin

% PRIOR TO ADDITION OF RED BLOOD CELL SUSPENSION

% AFTER ADDITION OF RED BLOOD CELL SUSPENSION

PERCENTAGE OF HUMAN SERUM IN PENICILLIN ASSAY
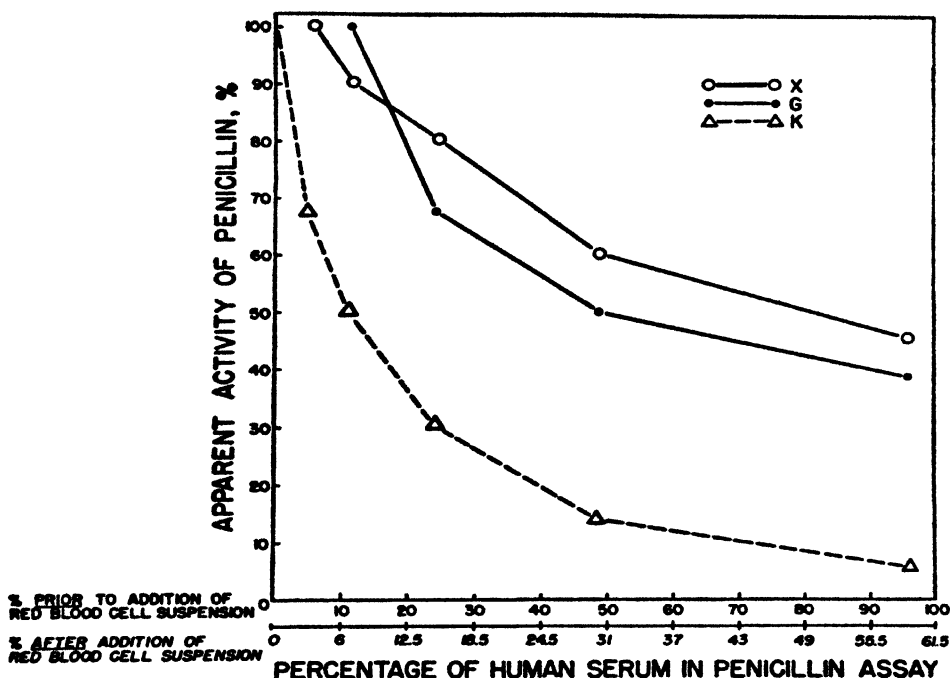
Figure 1. The effect of human serum on the bioassay of penicillins G, X, and K. Increasing the concentration of human serum diminishes progressively the *in vitro* activities of penicillins G, X, and K.

TABLE 2

The effect of human serum on the bioassay of penicillins G, X, and K: interpolated corrective factors

AMOUNT OF SERUM IN INDICATOR TUBE OF ASSAY,* ML	PERCENTAGE OF SERUM IN INDICATOR TUBE†		CORRECTIVE FACTORS (MEANS)‡		
	Before addition of red blood cells	After addition of red blood cells	G	X	K
0.8	Whole serum = 100	61.5	2.7	2.3	18.2
0.7	1:1.1 = 87.5	54	2.6	2.2	16.6
0.6	1:1.3 = 75	46	2.4	2.0	12.7
0.5	1:1.6 = 62.5	38.5	2.2	1.8	10.0
0.4	1:2 = 50	31	2.0	1.7	7.7
0.3	1:2.5 = 37.5	23	1.8	1.5	6.3
0.2	1:3 = 25	15.5	1.6	1.4	4.5
0.15	1:4 = 18.7	11.5	1.4	1.3	3.3
0.125	1:6.5 = 15.6	9.5	1.2	1.2	2.4
0.1	1:8 = 12.5	8	1.1	1.1	2.0
0.05	1:16 = 6.3	4	1.0	1.0	1.5
<0.05	1:32+ = <3	<2	1.0	1.0	1.0

* Smallest amount of serum which, brought up to a total volume of 0.8 ml and incubated with 0.5 ml of a human red blood cell suspension, completely inhibited hemolysis by the C-203 strain of *Streptococcus pyogenes.*

† For example, 0.1 ml of serum in total of 0.8 ml = 12.5 per cent; after the addition of 0.5 ml of red blood cell suspension used as the hemolytic indicator, serum concentration becomes 8.3 per cent.

‡ Factors by which the apparent penicillin concentration in the serum must be multiplied to compensate for the inhibitory effect of serum in the assay.

K, whereas 12 and 24 per cent appeared to be the limiting concentrations in the cases of penicillins X and G, respectively. In the presence of 96 per cent serum, penicillins K, G, and X were only 5.5, 35, and 46 per cent as active, respectively, as they were in its absence; and to compensate for that inhibition, the apparent activities would have to be multiplied by their reciprocals (18, 2.6, and 2.2, respectively) as *corrective factors*. These corrective factors, at a series of serum concentrations, are tabulated for each type of penicillin in the last columns of tables 1 and 2.

The practical aspects of these corrective factors lie in the fact that they can be used to compensate for the inhibitory effect of serum on the apparent penicillin content of a given serum specimen. Thus, in the dilution technique under present consideration, if the indicator tube of the assay (smallest amount of serum that inhibited hemolysis) contained 0.3 ml of serum in a total volume of 0.8 ml, the percentage of serum on the indicator tube, prior to the addition of the red blood cell suspension, was 0.3/0.8 = 37.5 per cent. As is indicated in table 2, for example, the corrective factor for penicillin K at that concentration of serum is 6.3. If the apparent penicillin content of the specimen was, e.g., 0.32 micrograms per ml, this value multiplied by the corrective factor of 6.3 gives as the actual penicillin concentration of the serum, corrected for the inhibitory effect of serum in the assay, the value of 2 micrograms per ml.

These findings are of primary interest to the investigator concerned with the pharmacology and mode of action of penicillin. For the physician, the uncorrected concentrations may be more useful since such values more nearly represent the concentrations available for distribution to the tissues *in vivo*.

It is apparent that these corrective factors can be applied retrospectively to previous studies in which the same assay technique had been used. For other methods of bioassay, it would be necessary empirically to derive similar tables of corrective factors.

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ORIGIN OF BACTERIAL RESISTANCE TO ANTIBIOTICS[1]

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In this brief review of the problem of the genetic aspects of the origin of bacterial resistance to antibiotics, I intend to discuss mainly work done in my laboratory. I shall (1) offer evidence that bacterial resistance to penicillin and streptomycin is not induced by these compounds but originates spontaneously through genetic changes comparable to gene mutations; (2) describe the resistance patterns observed in experiments with penicillin and streptomycin; and (3) outline a possible mechanism responsible for resistance, and for the differences between the resistance patterns observed with penicillin and those observed with streptomycin.

All experiments were done *in vitro*. Penicillin was tested with *Staphylococcus aureus*, strain NRRL 313 (Demerec, 1945a), and streptomycin was tested with the same strain of *S. aureus* and with *Escherichia coli*, strain B. The streptomycin was obtained from Chas. Pfizer & Co., New York, at a time when this compound was still very scarce, and I wish here to acknowledge their generosity. In all the experiments bacteria were first grown in broth cultures without any penicillin or streptomycin; these were used only in the tests for resistance, made by growing bacteria on broth agar plates containing various concentrations of one or the other antibiotic.

SURVIVAL CURVES

Figures 1 and 2 show the behavior of our strain of *S. aureus* when plated on nutrient agar plates to which specified amounts of penicillin (figure 1) or streptomycin (figure 2) had been added. It is evident from these curves that on low concentrations of either antibiotic all bacteria survived and formed colonies. Threshold concentrations are indicated on the curves by sharp breaks, the numbers of survivors decreasing very rapidly with increase of concentrations. The slope of the curves is very steep at concentrations near the threshold, but levels out as concentrations become increasingly higher. These curves show that we were dealing with mixed populations of types sensitive to antibiotics and types more or less resistant. Sensitive bacteria made up by far the largest portion of the populations.

The two curves are very similar in the region of lower concentrations. A striking difference is evident, however, in the region of higher concentrations; on the medium containing streptomycin, survivors continued to appear even at the highest concentration used in the experiments.

Tests of survivors confirmed the conclusion indicated by the survival curves. Strains isolated from the colonies of plates having high concentrations of either penicillin or streptomycin proved to be more resistant to the respective compound and grew on concentrations at least as high as those of the plates from which they
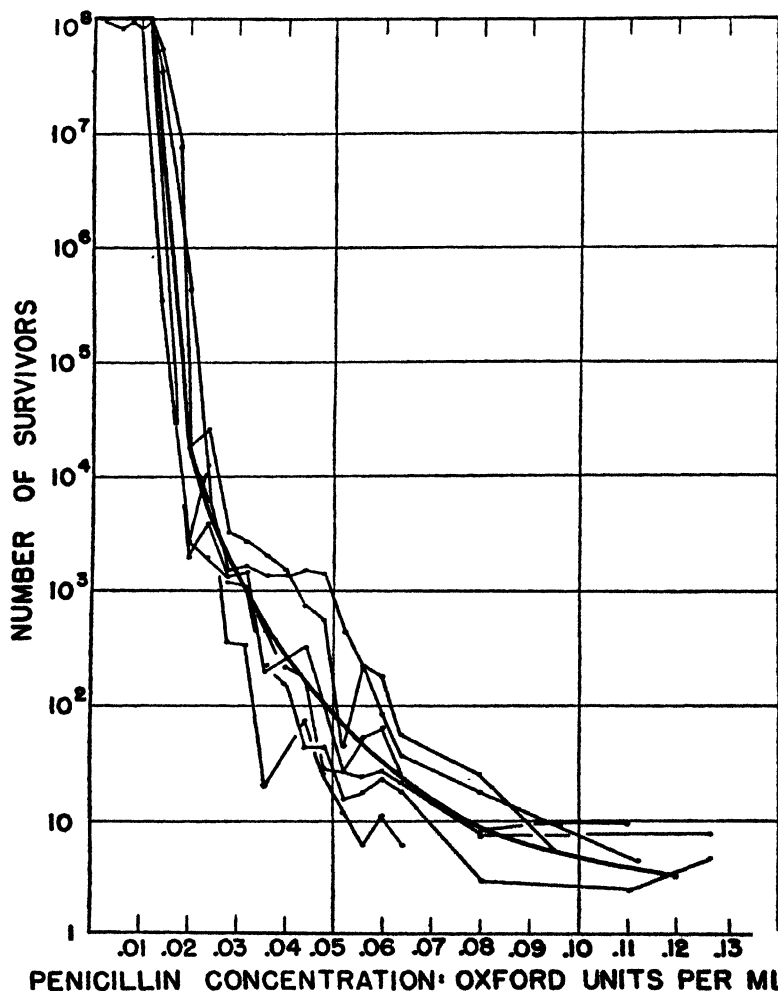
Figure 1. Survival curves for *Staphylococcus aureus* plated on nutrient agar containing various concentrations of penicillin. The six light curves represent results of six independent experiments, and the heavy curve represents the average of these experiments.

had been isolated. It was found that resistance to one of these antibiotics is independent of resistance to the other; that is to say, strains with increased resistance to penicillin are still sensitive to streptomycin, and vice versa. It seems unnecessary to discuss in detail the by now well-established fact that strains that once become resistant as a rule continue so.

ORIGIN OF RESISTANCE

The numerous experiments made in gathering the data for the survival curves showed clearly that in large populations of bacteria there were always some individuals more resistant to the antibiotics than others.   Since we used very small
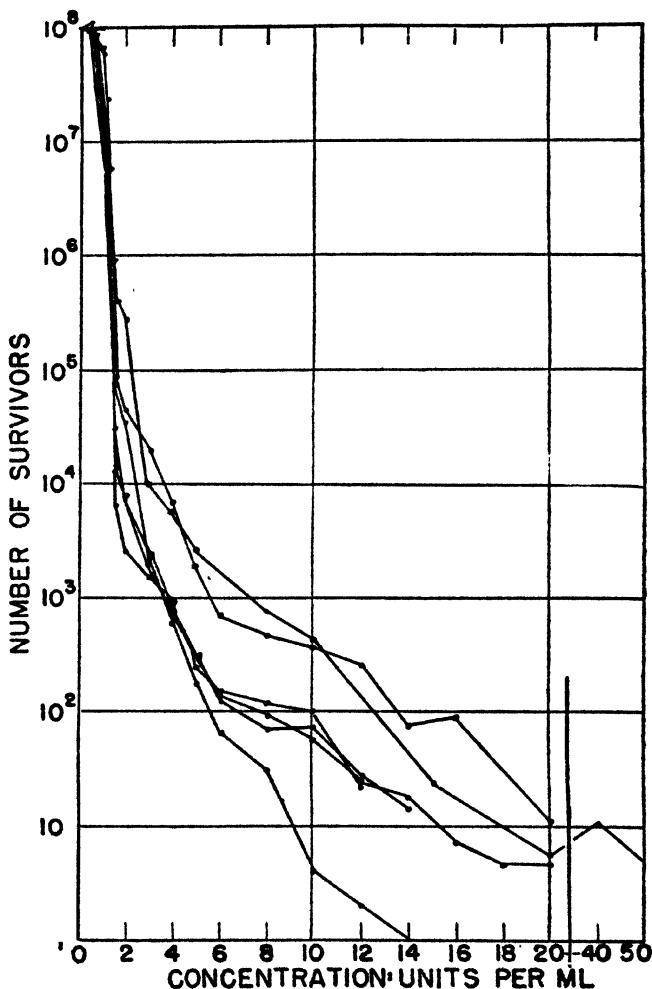
Figure 2.   Survival curves for *Staphylococcus aureus* plated on nutrient agar containing various concentrations of streptomycin.

inocula (50 to 300 bacteria), the proportion of resistant bacteria was too small to account for their presence by assuming that they came about through division of one or more resistant individuals that may have been present in the inoculum. Therefore the resistant individuals must have originated in the experimental cultures.   Two alternative possibilities were considered with respect to the

mechanism of this origin: (1) that resistance was induced by some interaction between the antibiotic and the bacteria when they were together on the plate; and (2) that it originated independently of the antibiotic, by mutation, the antibiotic acting only as a selective agent in the isolation of mutants by destruction of sensitive bacteria.

A relatively simple method was available for distinguishing experimentally between these two possibilities. It was devised by Luria and Delbrück (1943) in a study of the origin of bacterial resistance to bacteriophages, and was adapted for work with antibiotics in our study of the origin of resistance to penicillin (Demerec, 1945a). Following is a brief description of the method as used in our experiments.

From a single culture of bacteria, small inocula (50 to 300 bacteria) were taken and used to start 21 or more independent broth cultures. These were incubated for 24 hours, or until growth had reached the saturation point. During incubation the number of bacteria (in experiments using *E. coli* or *S. aureus*) increased to about 2×10^8 per ml. From the same culture that served as the source of the inocula, 10 samples of bacteria of the same size as the inocula were plated on a culture medium containing the same concentration of antibiotic as was used in the tests, in order to determine if any resistant bacteria were present in these samples. The concentration of the antibiotic had to be high enough so that there were no survivors among the small number of bacteria plated—in other words, that there were no bacteria resistant to that concentration in the inocula used to start cultures. It followed, then, that all resistant bacteria found in full-grown cultures had necessarily originated in these cultures during the period when the number of bacteria increased from 50 to 300 to about 2×10^8 per ml.

Next, from 20 of the broth cultures, samples of 0.1 ml were taken and plated on petri dishes containing the same concentration of the antibiotic (0.064 units of penicillin, or 5 units of streptomycin per ml of medium). Fifteen 1-ml samples were plated from the twenty-first tube. Thus two sets of plates were obtained: one set of 20 in which each plate had bacteria from a different culture, and another set of 15 all having bacteria from the same culture. The plates were incubated for 24 hours, or for a longer period if the growth of colonies was slow. After incubation the number of colonies on each plate was determined. These colonies represented resistant bacteria that had been present in the sample plated.

On both sets of plates in this test the experimental conditions were similar, like numbers of bacteria (about 2×10^7) having been plated onto nutrient agar containing identical concentrations of antibiotic. Therefore, if resistance were induced through interaction between the bacteria and the antibiotic when they were in contact with each other, approximately similar numbers of resistant bacteria would presumably be obtained on all the plates, regardless of the origin of the bacterial samples; the variation between plates should not exceed random variability. In the event that the origin of resistance is mutational, on the other hand, similar numbers of resistant colonies would be obtained only among the platings taken from the same culture, since these represented repeated tests of

the same mixture of resistant and sensitive bacteria. Among the samples from separate cultures, if mutations occur at random, a large number of resistant colonies would be obtained from cultures in which mutation happened to occur early in the growth of the culture, and a small number of resistant colonies from cultures in which mutation happened to occur late, provided the growth rate of resistant bacteria is not appreciably different from that of normal ones. If resistance originates by mutation, then, the variation in numbers of resistant bacteria would be much greater between samples taken from separate cultures than between samples taken from the same culture.

TABLE 1

Number of bacteria (E. coli) resistant to a concentration of 5 units of streptomycin per ml of agar medium in samples taken from a series of independent cultures and similar samples taken from a single culture which assayed 1.3 × 10⁹ bacteria per ml

SAMPLES FROM INDEPENDENT CULTURES				SAMPLES FROM SINGLE CULTURE			
Culture no.	No. of resistant bacteria	Culture no.	No. of resistant bacteria	Sample no.	No. of resistant bacteria	Sample no.	No. of resistant bacteria
1	67	11	56	1	142	11	110
2	159	12	91	2	155	12	125
3	135	13	123	3	132	13	135
4	291	14	97	4	123	14	121
5	75	15	48	5	140	15	112
6	117	16	52	6	146		
7	73	17	54	7	141		
8	129	18	89	8	137		
9	86	19	111	9	128		
10	101	20	164	10	121		
Average....................			105.9	Average....................			131.2
Variance...................			2913.9	Variance...................			151.1
Chi-square.................			550.3	Chi-square.................			17.3
P much less than..........			0.001	P.........................			0.26

Table 1 shows the results of such an experiment with *E. coli* and streptomycin. In addition to the tests represented in the table, the concentration of bacteria was determined in 11 cultures, including the one from which the 15 samples were taken. The average number of bacteria in 10 cultures was 2.2×10^9 per ml, with extreme variants of 1.9 and 2.3, and the average number in the eleventh was 2.1×10^9 per ml. Thus the variation in numbers of bacteria among the different cultures was so small that it could have introduced only negligible differences between the numbers of resistant colonies observed on different plates. It is evident from table 1 that the variation in number of resistant colonies was considerably greater among platings from independent cultures than among platings from a single culture. The extreme variants of independent cultures were 48 and 291, the average 106, the variance 2,914, chi-square 550, and the probability that this variation was due to chance is insignificant. On the other

hand, the variation in number of resistant colonies among platings of samples taken from one culture was very small; and the probability that this variation was due to chance is 26 per 100 trials. Very similar results were obtained in experiments using *S. aureus* and penicillin (Demerec, 1945a).

These results, then, favor the assumption that resistance to certain concentrations of penicillin or streptomycin originates through mutation, and that resistant bacteria may be found in any large population, the proportion depending on the mutation rate.

Oakberg and Luria (1947) reached identical conclusions after experimenting with *S. aureus* and sodium sulfathiazole. This suggests that mutations may be generally responsible for the origin of resistance that is transmitted to the offspring of the individuals that acquire it.

RESISTANCE STEPS

A very interesting feature of bacterial resistance to antibiotics is the stepwise increase in degree of resistance that can be brought about by selection. This feature is particularly well expressed in penicillin resistance. Figure 3 reproduces curves from an earlier paper (Demerec, 1945a) showing the effect of selection on the increase in resistance of *S. aureus* to penicillin. The first is the survival curve of the stock culture. At a concentration of 0.15 units per ml there were no survivors, but at a concentration of 0.12 units about 4 per 10^8 bacteria lived. First-step resistant strains were isolated from stock culture bacteria surviving sublethal concentrations. The second curve of figure 3 is a typical survival curve of such first-step resistant strains. Some individuals of these strains survived concentrations up to about 0.2 units. When first-step resistant strains were grown on sublethal concentrations of penicillin, second-step resistant strains were isolated from the survivors. A typical second-step survival curve is shown third on figure 3. Third- and fourth-step resistant strains were obtained in similar manner.

It is of interest to note that the building up of resistance is more rapid with each selection step. Thus, with our strain of *S. aureus*, a concentration of 0.15 units was sufficient to eliminate all bacteria of the original strain, but a concentration of about 0.2 units was required to eliminate all bacteria of the first-step resistant strain, and concentrations of about 0.4 units for the second-step, 1 unit for the third-step, and 7 units for the fourth-step. The fifth-step strain was for all practical purposes completely resistant to penicillin, since it was not affected by a concentration of 250 units per ml. With each step the increase in resistance appeared to be exponential.

Whereas the building up of resistance to penicillin followed a definite pattern, resistance to streptomycin showed a considerable degree of variability. Among first-step resistant strains—that is, among strains isolated from colonies of the original strain that survived sublethal doses of streptomycin—there were some that were only slightly more resistant than the original strain, some that were almost completely resistant, and some that fell between these two extremes (figure 4). It has been found that the variability in degree of resistance among

first-step penicillin-resistant strains (Demerec, 1945*b*) is slight as compared with the variability observed among first-step streptomycin-resistant strains.
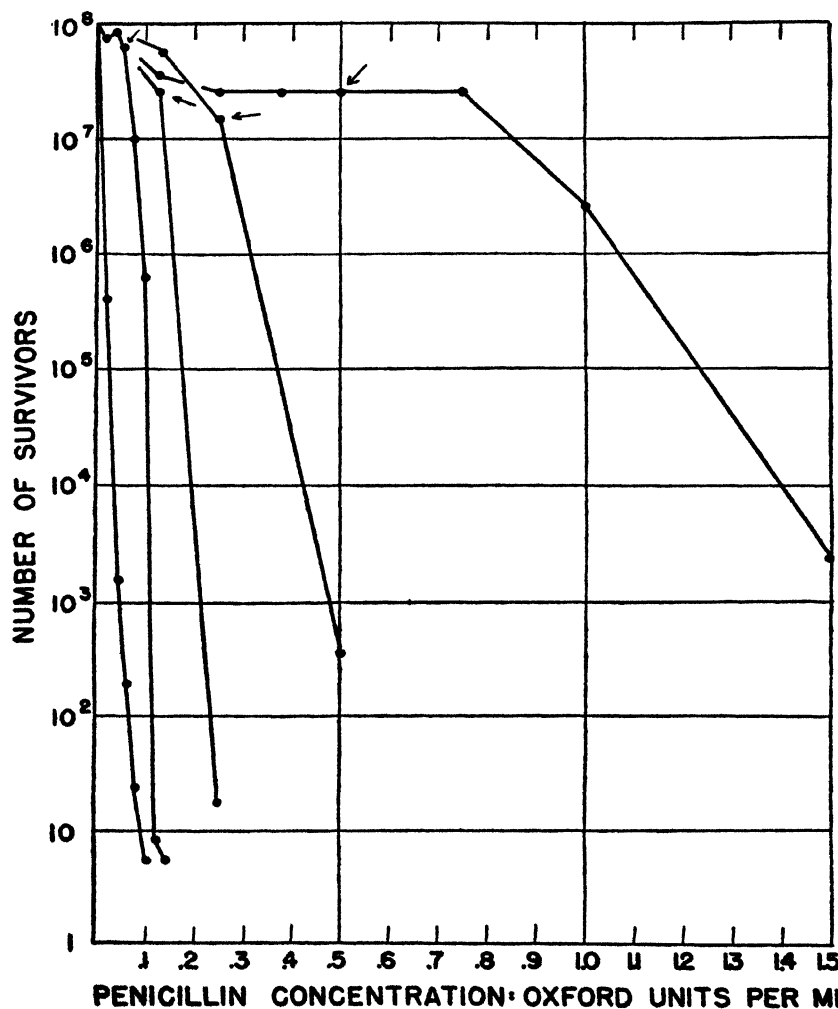
Figure 3. Stepwise build-up of resistance to penicillin in *S. aureus.* First from left, survival curve of stock culture; second, survival curve of first-step resistant strain isolated from a colony of the stock culture growing on the concentration indicated by the arrow. The other curves are of second-, third-, and fourth-step resistant strains isolated from colonies growing on the concentrations indicated by the arrows.

In the build-up of resistance to streptomycin, resistant strains of the second step, third step, etc., showed behavior similar to the first-step strains; that is, they exhibited a wide range of variability in degree of resistance. By plating bacteria of a low-resistance first-step strain on a concentration of streptomycin that is sublethal for that strain, one can isolate second-step resistant strains that

vary in degree of resistance from only slightly more resistant than the original strain to very resistant.   In fact, there is no difference in degree of resistance between the most resistant strains of the first step and those of the second, third,
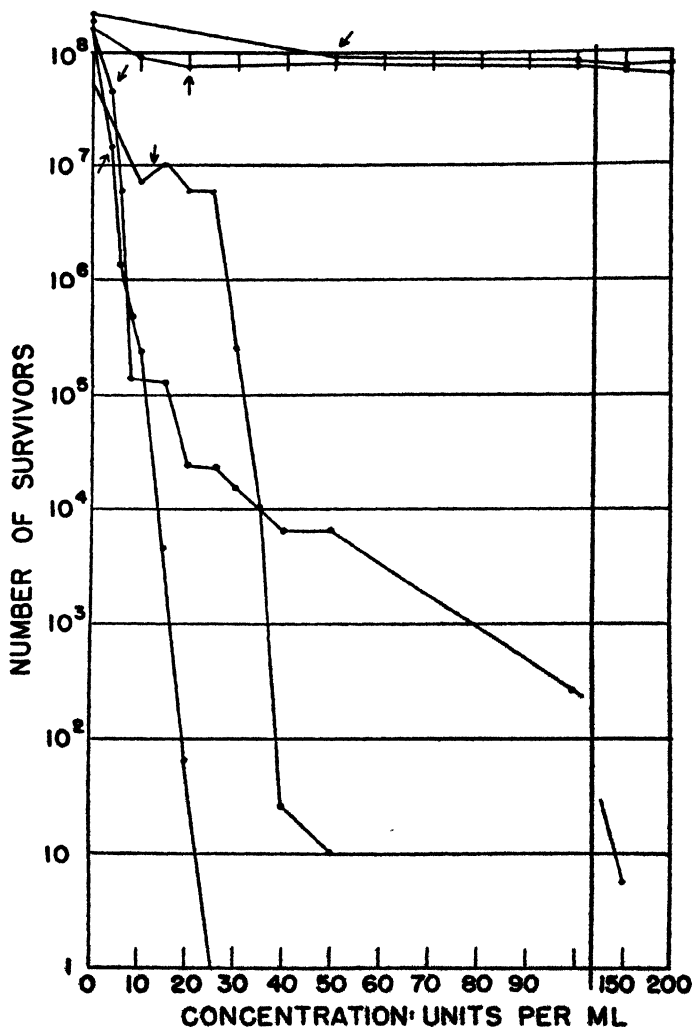
Figure 4. Survival curves of first-step strains of *S. aureus* resistant to streptomycin, showing the great variability in degree of resistance. Each strain was isolated from a colony of the stock culture growing on the concentration of streptomycin indicated by the arrow.

and higher steps. Consequently, strains that are highly resistant to streptomycin may be obtained either in one step, by selection of survivors of very high concentrations, or in several steps, by repeated selection of survivors from bacteria grown on increasingly higher concentrations.

POSSIBLE MECHANISM OF ORIGIN OF HIGH-DEGREE RESISTANCE

The experimental evidence available at present indicates that resistance to penicillin and resistance to streptomycin are independent of each other; that such resistance is a heritable property induced by genetic changes comparable to mutations; that first-step penicillin-resistant strains are fairly uniform in their degree of resistance, a highly resistant strain being built up by selection through several steps; and that first-step streptomycin-resistant strains show a great deal of variability in degree of resistance, highly resistant strains being produced either in one step by selection from among first-step resistant mutants or in several steps by repeated selection of strains having higher and higher degrees of resistance.

What mechanism is responsible for the stepwise build-up of resistance, and for the difference between penicillin resistance and streptomycin resistance in this regard? Evidence accumulated by several investigators in recent genetical research with bacteria makes it appear reasonably certain that mutations in bacteria are caused by changes in genes. Granting this assumption, the complexity of behavior observed in studies of resistance to antibiotics indicates that several genes must be involved. Such an inference is not new. Several years ago Demerec and Fano (1945) suggested that the complex situation observed in their study of resistance of *E. coli* to 7 phages pointed to the presence of about 20 distinct mutant types in their material. Since many more than 7 phages affect the strain of coli investigated, and since it is reasonable to assume that extension of the study to these phages would reveal additional mutants, it is evident that the genetic background of resistance to phages is very complex indeed, and that it involves a considerable number of genes.

If a like situation exists in respect to resistance to antibiotics, then it can be assumed that many genes are instrumental in determining resistance to the two antibiotics used in these experiments, and that the genes affecting resistance to penicillin are different from those affecting resistance to streptomycin. If any one of these genes should mutate, the bacterium in which such a mutation occurred and the strain developed from that bacterium would be more resistant to the respective antibiotic than was the original parent strain. Such a strain would be what we have called a "first-step resistant strain."

The fact that first-step penicillin-resistant strains are fairly uniform in degree of resistance (Demerec, 1945b, figure 2) is consistent with the assumption that all genes affecting resistance to penicillin have a similar potency, so that the effect of mutation is the same regardless of which of the genes happens to mutate. According to this hypothesis, there is still present in a first-step resistant strain a number of unmutated genes that affect resistance. Mutation of any of these produces a second-step resistant strain, which possesses a higher degree of resistance than the first-step strain. Similarly, by mutation of another gene in a second-step resistant strain, a still higher degree of resistance is attained, characteristic of the third-step resistant strain. From this, by further mutations, highly resistant fourth- and fifth-step strains may be obtained. The curves in figure 3 indicate that the increase in degree of resistance with each step is ex-

ponential. This means that the effect of two or more mutants together is considerably greater than would be expected from the added values of the effects of single mutants.

No attempt has been made to determine the frequency with which genes affecting resistance to penicillin mutate. It may be estimated from survival curves, however, that the mutation rate is low, in the neighborhood of 1×10^{-8}. With such a low mutation rate, it is evident that the increase in resistance must occur in successive steps, and that the chance that one step will be skipped is very slight, the chance that two steps will be skipped practically nil. (A step would be skipped when mutations in two genes occurred simultaneously in the same bacterium; and two steps would be skipped if three mutations occurred simultaneously in a single cell. The chance of two simultaneous mutations is $10^{-8} \times 10^{-8} = 10^{-16}$, and of three simultaneous mutations, $10^{-8} \times 10^{-8} \times 10^{-8} = 10^{-24}$. Since the volume of an *S. aureus* cell is about one cubic micron, it would be expected that one double mutant, on the average, would be found in ten liters of bacteria, and one triple mutant in one million cubic meters.)

The observed behavior of resistance to streptomycin also can be explained by assuming the existence of several genes determining such resistance. Unlike the genes for penicillin resistance, however, these differ greatly from one another in potency. If a gene of low potency mutates, the first-step resistant strain will have a low degree of resistance, but if mutation occurs in a highly potent gene, the first-step resistant strain will be highly resistant. Consequently, considerable variation in degree of resistance is to be expected between first-step strains; and for the same reason a highly resistant strain may be obtained either in one step, by selection of a highly resistant first-step mutant, or in several steps, by selection of mutants of low resistance values.

CLINICAL CONSIDERATIONS

A major consideration in the clinical use of antibiotics is how to avoid the development of resistant strains, since the usefulness of an antibiotic is closely related to the number of resistant pathogens and to their incidence in infections. For this reason, analysis of the mechanism of origin of resistance to penicillin and streptomycin has an important bearing on the clinical application of these antibiotics.

From the clinical standpoint, the situation in regard to penicillin is relatively simple and well defined. Since resistance develops in steps, and it is very unlikely that a step will be skipped in the process, the clinician can avoid development of resistant pathogens by using initial doses that are adequate for the elimination of first-step resistant individuals. Fortunately, most of the common pathogenic strains that have been investigated (North and Christie, 1945; Meads et al., 1945) are very sensitive to penicillin, so that large doses are not required in clinical use. It is equally important for the clinician to maintain the effective concentration in treatment as long as the infection persists, because decrease of the concentration below the effective level will permit the accumulation of first-step resistant bacteria, which may increase to a point that will allow the occurrence of

second-step mutants. These are difficult to control. If there is any suspicion that a pathogen may be more resistant to penicillin than the usual strains, it is advisable to determine the degree of resistance before starting treatment, and to adjust the concentration accordingly. If adequate precautions are taken against the development of second-step resistant bacteria, there should be no danger from resistant pathogens in penicillin treatment. It is particularly important to avoid the indiscriminate use of penicillin, however, especially for applications where it can scarcely be of any help (for example, as a mouth wash), because there is positive danger that such use may stimulate the development of resistant strains.

In the clinical use of streptomycin, the situation can be controlled to a much smaller extent. Since highly resistant bacteria are found among the first-step mutants, treatment with high concentrations is not effective in eliminating the whole population of bacteria present in an infection. What it does accomplish is a reduction of the number of bacteria to a level with which the organism is capable of dealing. If for some reason the organism cannot do this, the chances for the development of a resistant strain are exceedingly good. Therefore, it must be expected that pathogenic strains resistant to streptomycin will frequently develop, in the course of time replacing sensitive strains in communities where streptomycin is used and rendering this antibiotic ineffective. This eventuality can be postponed by restricting the use of streptomycin to serious infections which cannot be controlled in any other way.

It may be appropriate to mention here the most effective way, theoretically, of preventing the origin of resistant strains of bacteria. This is the use in clinical treatment of a mixture of two antibiotics, when such are available, that affect the same pathogen but are independent in their actions. The evidence of independence is that bacterial strains that have developed resistance to one antibiotic are still sensitive to the other, and vice versa. If such a mixture of two antibiotics is used, then only bacteria that are resistant to both can survive the treatment and form first-step resistant strains. Such bacteria would be exceedingly rare. For example, if first-step resistant bacteria for each of two antibiotics should be found in a large population with a frequency of 1×10^{-7}, then the expected frequency of bacteria resistant to both these antibiotics would be 1×10^{-14}.

SUMMARY

A method is described that has been used to determine whether resistance to streptomycin is induced by interaction of the compound with bacteria or originates by gene mutation. Data are presented indicating that mutations are responsible for the origin of streptomycin resistance in *Staphylococcus aureus*. These agree with previously published data regarding the origin of penicillin resistance in the same organism.

The stepwise increase of resistance to penicillin by selection is explained by assuming that mutations in several equally potent genes are effective in inducing resistance, and that the slight degree of resistance characteristic of the first step is due to a mutation in one of these genes, the higher degrees of resistance of subsequent steps to successive mutations in other genes.

The increase in resistance to streptomycin also can be explained by the assumption that several genes are instrumental in the process. These genes vary greatly in their potency, however, and consequently a mutation in a highly potent gene will be responsible for a high degree of resistance, a mutation in a less potent gene for a low degree of resistance.

From the knowledge gained concerning the mechanism of origin of resistance, it is concluded that in treatment with penicillin the development of highly resistant strains can be avoided by application of the penicillin in doses sufficiently large to prevent survival of first-step resistant mutants. In treatment with streptomycin, however, the development of highly resistant strains cannot be prevented; effective treatment does not eliminate all bacteria, but it probably reduces their number to a level at which the organism is able to eliminate them.

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THE ACTION OF PENICILLIN ON STAPHYLOCOCCUS: FURTHER OBSERVATIONS ON THE EFFECT OF A SHORT EXPOSURE

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When a culture of *Staphylococcus* in the logarithmic period of growth is exposed to penicillin in appropriate concentration, growth of the bacteria is stopped promptly. If within a short time the penicillin is removed, no decrease in population of viable cells occurs, but, after a lag period, growth is resumed (Parker and Marsh, 1946). In earlier work it was shown that this effect occurred even though there was no detectable killing of organisms; it was not a peculiarity of the metabolic state of "persisters" (Bigger, 1944) in the culture. In the experiments to be detailed in this report, the observations have been extended to cover a total of 29 strains of *Staphylococcus* in order to determine whether the phenomenon was unique in the strain first observed or whether it was generally distributed. The strains were isolated from clinical material in a hospital laboratory and exhibited a wide range of penicillin sensitivity.

Source and selection of cultures. In the course of a study of the relation between size of the test inoculum and apparent resistance to penicillin, all strains of *Staphylococcus aureus* isolated from clinical material in the routine laboratory serving Lakeside Hospital were subjected to tests of penicillin sensitivity (Parker, 1946). From the 169 cultures so studied, 29 were chosen, covering the range of sensitivity exhibited by the series.

Test of sensitivity. The sensitivity test used was that described previously (Parker and Marsh, 1946; Parker, 1946). Serial dilutions of penicillin were prepared in nutrient broth; each was inoculated with 0.5 ml of a 10^{-6} dilution of an overnight broth culture. The tubes were incubated overnight, and the tube was then identified which contained just enough penicillin to prevent visible growth of bacteria. The final volume of the test was 1 ml. This was designated as the "penicillin sensitivity (small inoculum)" of the strain. Another test was made simultaneously, which differed only in that the inoculum contained 10,000 times as many organisms, and the inhibitory penicillin concentration identified in this test was designated as "penicillin sensitivity (large inoculum)."

Medium. The medium used was the tryptose broth supplied by the Digestive Ferments Laboratories; the pH after sterilization adjusted to 7.4 to 7.6.

EXPERIMENTAL RESULTS

In the first studies on this subject it was found, as it had been by others (Rantz and Kirby, 1944; Hobby and Dawson, 1944), that at very low concentrations of penicillin there was a definite interval between the time of addition of penicillin

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[1] With the technical assistance of Helen Ferguson.

and the first demonstrable decrease in the number of viable bacterial cells, multiplication apparently continuing for a short time in the presence of the low concentration of penicillin.   With higher concentrations, multiplication was apparently checked promptly, and replaced by a logarithmic decline in bacterial numbers.   In the present experiments the duration of the initial stationary period of the culture on inoculation of an 18-hour culture into a new medium was determined, and also the time after inoculation when the population had increased by about 100-fold.   Then, in order to determine the effect of brief exposure to penicillin, a culture was prepared that was in the logarithmic phase of growth and that had a population of about 10^4 viable bacteria per ml.   A predetermined amount of penicillin was added.   After 15 minutes the bacteria were
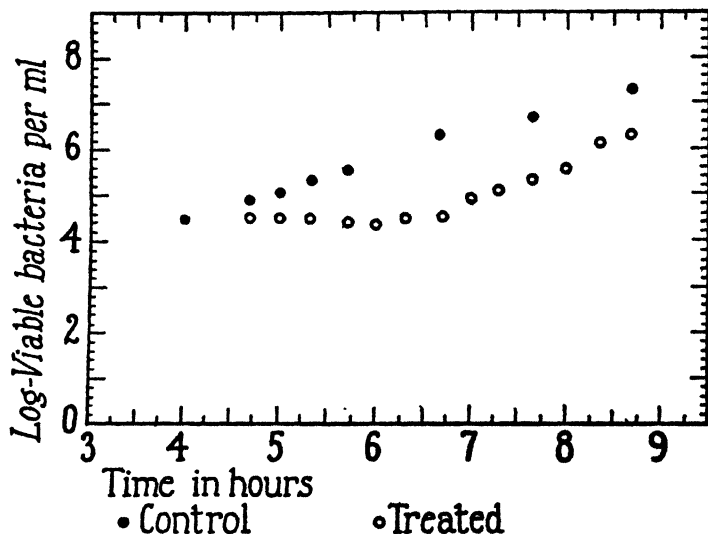
Figure 1.   Effect of short exposure of Staphylococcus to penicillin.   A 4-hour culture, exposed to penicillin for 15 minutes, then centrifuged and cells transferred to penicillin-free broth.

removed to fresh penicillin-free broth, and the culture was observed until growth was resumed.   The details of technique were as described below.

To 50 ml of tryptose broth, previously brought to 37 C, enough of an overnight (18-hour) culture was added to give a final count of about 100 cells per ml.   One-ml samples were withdrawn at 20-minute intervals thereafter for 6 hours, and plate counts of viable cells were made.   From the data so obtained the time was determined at which the culture would be expected to contain 10,000 cells per ml, and a similarly prepared culture of this age was used in subsequent experiments. The exact time varied but was usually about 4 hours from the time of first inoculation of the flask.

The effect on the organism of continuous exposure to penicillin was next determined.   To the growing culture (4 hours after inoculation), penicillin was added in a quantity adequate to produce as a final concentration a small multiple of the
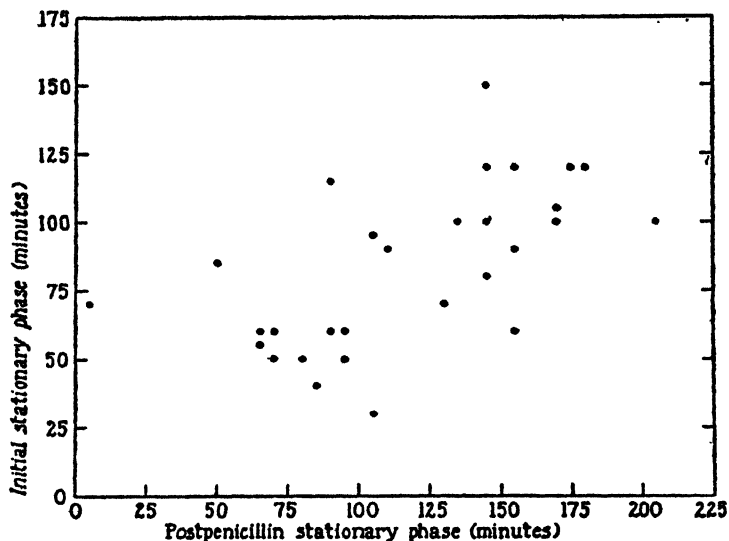
Figure 2. Scatter diagram. The relation between initial stationary phase and post-penicillin stationary phase of growth, both in minutes.
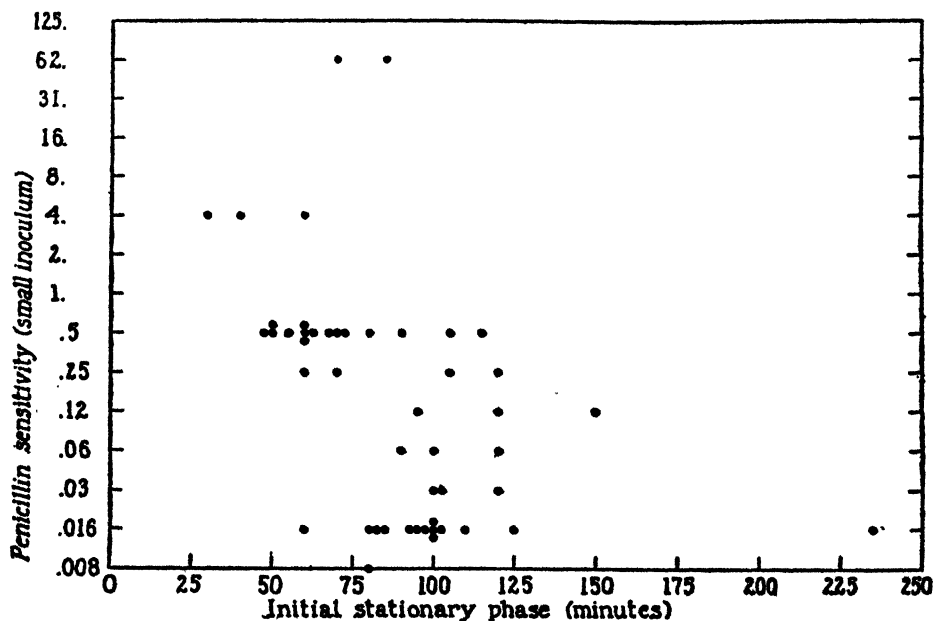
Figure 3. Scatter diagram. The relation between penicillin sensitivity (small inoculum) and initial stationary phase of growth in minutes.

previously determined inhibitory concentration (small inoculum). At 20-minute intervals thereafter aliquot portions were withdrawn, the cells centrifuged down

and resuspended in fresh medium in order to remove the penicillin, and dilutions made for plate counts. The lowest concentration that served to check multiplication promptly, initiating a progressive fall in numbers of viable bacteria, was designated as the "minimal bactericidal concentration" and used in the short exposure experiments.

TABLE 1

Effect of short exposure to penicillin on Staphylococcus

CULTURE NO.	SENSITIVITY, SMALL INOCULUM*	SENSITIVITY, LARGE INOCULUM*	"BACTERICIDAL CONCENTRATIONS"†	INITIAL STATIONARY PERIOD	SECONDARY STATIONARY PERIOD
	units/ml	*units/ml*	*units/ml*	*minutes*	*minutes*
1	0.06	0.06	8	100	145
2	62	250	400	70	5
8	0.25	500	8	105	170
9	0.03	0.03	0.5	120	155
16	0.25	500	8	120	175
25	0.06	0.06	1	120	180
31	0.5	250	32	90	110
37	62	125	400	85	50
48	0.25	2	3	60	155
54	0.016	0.25	0.5	100	205
68	0.5	125	32	115	90
70	0.12	500	2	150	145
76	0.016	0.016	0.25	100	170
81	4	8	250	40	85
85	4	8	250	30	105
91	0.12	500	2	120	145
114	0.5	31	32	80	145
126	0.25	125	32	70	130
136	0.06	8	0.5	90	155
140	0.125	0.125	8	95	105
142	4	8	250	60	95
146	0.016	0.06	0.125	100	135
124	0.5	125	4	50	70
101	0.5	125	4	50	95
107	0.5	125	4	55	65
113	0.5	250	2	60	90
115	0.5	125	2	60	70
116	0.5	250	2	60	65
131	0.5	125	2	50	80

* Sensitivity to penicillin inhibition in overnight test. "Small inoculum" consists of 0.5 ml of 10^{-4} dilution of culture. "Large inoculum" is a 10^{-2} dilution.

† Concentration of penicillin inducing a prompt fall in population when added to a growing culture.

In determining the effect upon *Staphylococcus* of short exposure to penicillin, a culture was prepared as described above. At the predetermined time after inoculation, when the concentration of viable cells would be expected to be about 10,000 per ml, penicillin was added to one portion of the culture, in a quantity sufficient to give the "minimum bactericidal concentration." A second portion was reserved for control. (These manipulations were conducted in a 37 C room.)

After 15 minutes both treated and control cultures were removed from the warm room and centrifuged for 5 minutes, the supernatant was replaced with warm penicillin-free broth, and the cultures were again centrifuged. The supernatant was removed and the sedimented cells were suspended in a volume of warm broth equal to the original and replaced in the 37 C room. At intervals samples were taken from each and plate counts made to determine the number of viable cells.

The results of such an experiment are portrayed graphically in figure 1. It will be seen that the manipulations are almost without effect on the control culture. In contrast, the culture exposed to penicillin enters a second stationary phase of growth which, after a period, is succeeded by a logarithmic period of multiplication. Pertinent data for all of the cultures studied are given in table 1, and diagrams illustrating the correlation of certain values are given in figures 2 and 3.

SUMMARY AND DISCUSSION

The present experiments are seen to confirm and extend the previous observations on the effect of brief exposure of *Staphylococcus* to penicillin. Exposure of a growing culture to an appropriate concentration for 15 minutes followed by removal of the penicillin is consistently followed by a period during which the population of viable cells is constant. This effect is not due to selective killing of dividing cells, leaving resting cells unaffected, for the effect is as apparent when there is no change in the viable cell count after penicillin treatment as when the population is moderately reduced. It was anticipated when the experiments were undertaken that there might be a correlation between the penicillin-induced lag period and the ability of the culture to dispose of penicillin with penicillinase. Other experiments (Gilson and Parker, 1948) have shown that no correlation exists between the duration of the postpenicillin lag period of a culture and its content of penicillinase. There is, however, a high degree of correlation between the duration of the postpenicillin lag period, on the one hand, and both the duration of the initial lag period (figure 2) and the resistance to penicillin (figure 3), on the other. The significance of these associations is not immediately apparent. It is seen from inspection of figure 4, portraying data from a larger number of strains, that a relation exists between the degree of penicillin sensitivity and the duration of the initial lag period. The degree of association between these two values for the 29 strains here reported, which include all of the highly sensitive and all of the highly resistant strains available, is much less than that existing between the penicillin sensitivity and the duration of the postpenicillin dormant period, but is nevertheless significant. The coefficient of correlation is 0.8, indicating that the probability that such an association as is seen here could occur by chance alone is much less than 0.01.

In the absence of complete knowledge of the metabolic basis for the initial lag period, and lacking precise information on the mechanism of action of penicillin, it may be fruitless to speculate on the significance of the lag period that is induced by brief exposure to this substance. Several hypotheses, however, suggest themselves. It may be that penicillin blocks the synthesis of some material essential

for the growth of the cell; Krampitz and Werkman (1947) have suggested that this is ribonucleic acid. If this be so, it may be that while synthesis of this substance is blocked, the metabolism of other materials proceeds, and the supply of some other essential metabolite is exhausted. With release of the penicillin inhibition, time would be required for the resynthesis of this other metabolite before growth could proceed. The time required for a cell to repair such drug-produced metabolic damage might be related to its ability to repair the damage produced by aging, which some believe to be responsible for the initial lag period. It is conceivable also that penicillin combines with or otherwise renders useless some essential metabolite. If this were true, the postpenicillin lag period would be accounted for as the time required to synthesize a new supply of the metabolite in question before growth could proceed. On the other hand, the relation be-
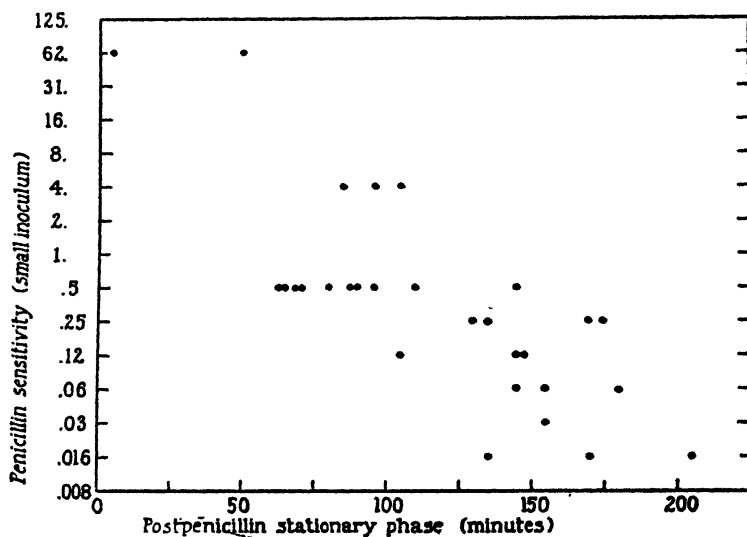
Figure 4. Scatter diagram. The relation between penicillin sensitivity (small inoculum) and postpenicillin stationary phase in minutes.

tween the length of the lag period and the sensitivity of the strain to penicillin suggests that the explanation may be simpler. It has been shown that penicillin in aqueous solution is adsorbed by certain serum proteins, and it is possible that it might be similarly adsorbed by bacterial proteins. Donovick, Lapedes, and Pansy (1947) explain the results obtained by them in testing the effect of mixtures of penicillins as probably due in part to differential avidity of adsorption of the different compounds by the bacterial cell. If it be assumed that such adsorbed penicillin could act to interfere with some essential process such as ribonucleic acid synthesis, it would appear to be not unreasonable to predict that the sensitivity of the organism might vary with the firmness with which penicillin is bound, and that in turn the removal of penicillin on transfer to a penicillin-free medium would have an inverse correlation with sensitivity. The data of figure 4 indicate that among the other factors correlated with the resistance of an organ-

ism to penicillin is the ability of the "resting" or more properly the "aged" cell quickly to become a "growing" cell. Thus a cell in which these adjustments are made slowly is in general more sensitive to penicillin effect than one with a more rapidly acting mechanism. This explanation appears to be consistent with the data recorded above, and experiments designed to test it as well as other hypotheses are now in progress.

These experiments may also have a bearing on the theoretical basis for the practice of chemotherapeutics. It is widely held that best results in chemotherapy with penicillin are achieved when the penicillin concentration is held constant, and many ingenious devices have been invented to facilitate this. The experience of the clinic, however, is witness to the general effectiveness of intermittent injections, often given in doses that allow the concentration of penicillin to fall below theoretical inhibiting levels for part of the time. The data presented may provide a partial explanation. Reference to figure 3 reveals that when staphylococci having a penicillin sensitivity of 0.25 units per ml or less (87 per cent of our strains) are exposed for 15 minutes to a penicillin concentration of the order of 1 unit per ml *and the penicillin then removed*, no bacterial multiplication occurs for (on the average) 2½ hours. Penicillin serum concentrations of this order may be maintained for 15 to 30 minutes after intramuscular injection of 50,000 units. Further, the penicillin is not then immediately removed, but its concentration falls slowly. If bacterial reactions *in vivo* are similar to those we have observed, at least part of the reason for the paradoxical efficiency of intermittent administration of penicillin may be at hand. In this connection it is to be remembered that all of the observations under discussion were made on *Staphylococcus*, and may not be transferable without modification to other genera.

CONCLUSIONS

Multiplication in a young culture of *Staphylococcus*, stopped by brief exposure to an appropriate concentration of penicillin, is resumed after a variable period of incubation in a penicillin-free medium.

The duration of the "postpenicillin stationary phase" is directly related to the initial lag period of growth, and to the penicillin sensitivity of the strain.

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PANCREATIC DIGEST CHOCOLATE BLOOD AGAR FOR THE ISOLATION OF THE GONOCOCCUS

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Since *Neisseria gonorrhoeae* was first cultivated by Bumm (1885) on placental blood serum, beef serum, or sheep serum, many media have been devised and tested for the isolation of the gonococcus from suspected cases of gonorrhea. Prior to 1933 the medium most used was the Douglas (1914) agar. This was a tryptic digest of beef enriched with either ascitic fluid, hydrocele fluid, beef serum, or defibrinated blood, and frequently with the addition of 1 per cent glucose. In 1910 Cohen and Fitzgerald first introduced the use of chocolate blood agar for the isolation of *Hemophilus influenzae*, but it was not until 1927 that McLeod and his associates used chocolate agar for the isolation of the gonococcus. The chocolate agar was prepared either by using a base of "filtered, sterilized, 75 C meat extract" or by using Wright's bouillon, which is made by extracting meat and peptone together.

Wherry and Oliver (1916) reported that the gonococcus grew best when grown in an atmosphere of approximately 10 per cent carbon dioxide. This was again recommended by McLeod (1934), although Erickson and Albert (1922), Cook and Stafford (1921), and Torrey and Buckell (1922) had considered reinforcing the air with carbon dioxide to be unnecessary for the isolation of the gonococcus.

Leahy and Carpenter (1936), using chocolate agar prepared from a Douglas agar base and incubating cultures for 48 hours at 37 C in an atmosphere containing 10 per cent carbon dioxide, isolated gonococci in 34 per cent of 302 specimens. By using the oxidase test, 14 per cent of 146 cultures that would otherwise have been reported negative were found to be positive. Also by incubating cultures in 10 per cent carbon dioxide they were able to isolate 15 per cent more positive cultures than were recovered from duplicate cultures incubated aerobically, under conditions that were otherwise identical.

Finger, Ghon, and Schlaganhaufer (1894) stated that the gonococcus grew best in a slightly acid medium. They used litmus as an indicator and the degree of acidity was not determined. Swartz, Shohl, and Davis (1920) showed the acid end point of the gonococcus, *in vitro*, to be pH 6.6.

The report to be presented in this paper comprises a study of the value of pancreatic digest of beef heart as described by Brown (1948) for supporting the growth of the gonococcus in pure culture and as a medium for the isolation of the organism from cervical secretions.

STUDIES ON THE GROWTH OF PURE CULTURES OF NEISSERIA GONORRHOEAE IN
PANCREATIC DIGEST BROTH AND ON PANCREATIC DIGEST
CHOCOLATE BLOOD AGAR

Six freshly isolated cervical strains of the gonococcus were tested for their
ability to grow in pancreatic digest broth having various pH values and on
pancreatic digest chocolate blood (human, 10 per cent) agar, pH 7.2. A series
of tubes containing 5.0 ml of the broth to be tested and 0.1 ml of a 24-hour broth
culture (pH 7.2) were incubated at 37 C for 24 hours. The tubes were then
thoroughly shaken and 0.1 ml from each tube was transferred to a corresponding
pancreatic digest chocolate blood agar plate. The plates were spread, incubated
for 24 hours, and the colonies counted.

The six strains reacted in the same manner. Prolific growth was obtained in
broth at pH 7.2 as evidenced by innumerable colonies per plate. At pH 6.8 the
growth was reduced approximately 50 per cent as compared with growth in broth
at pH 7.2. At pH 6.6, 6.4, 6.0, and 5.8 all plates were sterile. A duplicate set
of plates was made and incubated in an atmosphere of approximately 10 per cent
carbon dioxide. The results were the same as described above.

After 48 hours' incubation the colonies are quite characteristic. They are of
medium size, round, smooth, convex, and have glistening "wet"-appearing
surfaces. The colonies have a tendency to stick to the needle, forming threads,
when being transferred.

STUDIES ON PANCREATIC DIGEST CHOCOLATE BLOOD AGAR FOR THE ISOLATION OF
NEISSERIA GONORRHOEAE FROM CERVICAL SECRETIONS

This study comprises a total of 580 cervical cultures from 411 patients and a
total of 452 cervical smears from 283 patients. All plates were incubated for 48
hours at 37 C in an atmosphere of approximately 10 per cent carbon dioxide.
Oxidase tests were routinely made on all plates.

Group I. The results of cervical cultures from dispensary patients suspected of
having gonorrhea. The cases are divided according to diagnosis.

```
        Total number of cultures........................................ 405
            Positive cultures........................ 153 (37.7 per cent)
            Negative cultures.         .  ............ 252 (62.3 per cent)

        Acute gonorrhea
            Total number...............................  ..................... 86
                Positive cultures...................... 37 (43.0 per cent)
                Negative cultures..... ............. . 49 (57.0 per cent)

        Urethritis
            Total number...............................  ........................... 13
                Positive cultures..................... 8 (61.5 per cent)
                Negative cultures..... ..... ......... 5 (38.5 per cent)

        Cervicitis
            Total number........ .  ......  ..................... 155
                Positive cultures....... ... .......... 64 (41.2 per cent)
                Negative cultures........ ... . ..... 91 (58.8 per cent)
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Salpingitis
    Total number.. 125
        Positive cultures...................... 37 (19.5 per cent)
        Negative cultures...................... 88 (80.5 per cent)

Pelvic inflammatory disease
    Total number......................... 26
        Positive cultures...................... 7 (26.9 per cent)
        Negative cultures...................... 19 (73.1 per cent)

Group II. This series comprises cervical cultures from ward and dispensary patients, all having clinical and physical signs of acute gonorrhea. At the time of culturing, the pH of the cervical mucus was tested by using a series of buffer solutions and indicators as described by Brown (1924). The following results were obtained:

    Total number of cultures 175
        Positive cultures............. . .... 113 (64.2 per cent)
        Negative cultures.. .... 62 (35.8 per cent)

    Cultures taken when the cervical mucus pH range was 7.5 to 6.7
        Total number...... 129
        Positive cultures. .... 109 (86.0 per cent)
        Negative cultures .... 20 (14.0 per cent)

    Cultures taken when the cervical mucus pH range was 6.6 to 5.2
        Total number 46
        Positive cultures..................... 4 (8.7 per cent)
        Negative cultures........ .... 42 (91.3 per cent)

A comparison of the method of cervical smear examinations and cervical cultures. From groups I and II a total of 452 cervical smears were examined. All smears were stained by Burke's modification (1922) of the gram stain. In this series 96 or 21.2 per cent of the smears were positive, whereas the gonococcus was isolated from 230 or 50.8 per cent of the same group of patients.

<div align="center">DISCUSSION</div>

When pancreatic digest chocolate blood agar was used for the isolation of the gonococcus in a series of 580 cervical cultures, 266 or 45.9 per cent were positive. When the patients were divided into two groups, the group having only cultures (405) yielded 153 or 37.7 per cent positive cultures. The group having the pH of the cervical mucus tested at the time of culturing (175) yielded 113 or 64.2 per cent positive cultures. This simply means that if cultures are taken when the pH range of the cervical mucus is 7.5 to 6.7 (the optimal growth range of the organism) the chances of isolating the gonoccocus are greatly increased. Conversely, if cultures are taken when the pH of the cervical mucus is acid (6.6 to 5.2), the chances of isolating the organism are greatly decreased. It is shown by the figures that, in a total of 62 negative cultures in this group, 42 or 67.7 per cent were obtained when the pH was 6.6 and below, pH 6.6 corresponding to the

acid death point of the gonococcus when grown *in vitro*. Many of the patients who were again cultured during the estrogenic phases of their subsequent menstrual cycles yielded positive cultures (Koch, 1947).

In the group of patients having cervical smear examinations and cultures, the cultural method resulted in the discovery of 29.6 per cent more gonococcal infections than by the use of smear examinations alone. This should re-emphasize the invalidity of negative smears.

ACKNOWLEDGMENT

The author wishes to thank Dr. J. Howard Brown for his valuable assistance during the course of this investigation.

SUMMARY

Pancreatic digest chocolate blood agar supported a prolific growth of the gonococcus in pure culture after 24 hours' incubation at 37 C, both aerobically and in an atmosphere containing approximately 10 per cent carbon dioxide.

The medium proved to be of great value in the isolation of the organism from cervical secretions, especially in cases of acute gonorrhea from which 57.4 per cent positive cultures were obtained.

Negative cervical cultures do not indicate the absence of foci of infection or the lack of a sufficiently favorable medium upon which to isolate the organism. Other factors influencing negative cultures may be involved, an important one being the pH of the cervical mucus at the time of culturing.

The cultural method for the diagnosis of gonococcal infections is far superior to smear examinations.

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GROWTH OF THE COLI-AEROGENES GROUP IN THE PRESENCE OF ISOCITRATE

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Some time ago during study of the utilization of organic acids by the coli-aerogenes group it was noted that *Escherichia coli* of intestinal origin failed to grow in a simple medium containing citrate as the only organic compound, but *Aerobacter aerogenes*, in contrast, grew readily (Koser, 1923, 1924). Other compounds with some structural similarity to citric acid, such as tricarballylic, aconitic, glutaric, and adipic acids, did not permit the same differentiation between the coli and aerogenes types (Koser, 1926).

TABLE 1

Growth in citrate and isocitrate media

	Citrate 24 hr	Isocitrate		
		GROWTH AT 30 C		
		24 hr	48 hr	72 hr
E. coli....	0	0	0	0 (19)
A. aerogenes.............. .	++++	+	++	+++ (7)
		0	0	0 (13)
Intermediate (*Citrobacter*)....	++++	+	++	+++ (2)

Figures in parentheses at right show number of cultures tested. 0 indicates a clear tube. The number of plus signs denotes increasing turbidity, with ++++ the heaviest growth.

Recently an opportunity was afforded to test the behavior of organisms of the coli-aerogenes group toward isocitric acid, a compound that was not available earlier. A sample of the dimethyl ester of isocitric acid lactone, isolated from *Bryophyllum* leaves, was obtained through the kindness of Dr. H. B. Vickery of the Connecticut Agricultural Experiment Station. To convert this compound to the sodium salt of isocitric acid it was dissolved in water, a slight excess over the calculated quantity of sodium hydroxide was added, and the mixture was warmed on a steam bath for 15 minutes to saponify the ester and open the lactone ring (Pucher, Abrahams, and Vickery, 1948). This gives the sodium salt of the natural optically active isocitric acid, or sodium D-isocitrate. The solution was sterilized by passing it through a sintered glass filter.

The basal medium used for the tests was similar to that of the earlier work and consisted of 0.5 per cent sodium chloride, 0.1 per cent dibasic potassium phosphate, 0.1 per cent ammonium acid phosphate, and 0.02 per cent magnesium sulfate. To one portion of this, 0.3 per cent sodium citrate was added; to the

other portion, 0.3 per cent sodium isocitrate. The pH of both media was 6.8 to 6.9 after addition of the compounds.

Results with 19 *E. coli* cultures isolated from human and animal feces, 20 *A. aerogenes* cultures from soil, and 2 "intermediate" or *Citrobacter* cultures (methyl red +, Voges-Proskauer —, citrate +) from soil are presented. In table 1 the growth shown by plus signs is the average for each type. Some individual cultures grew a little more rapidly or more slowly than the average.

All fecal *E. coli* failed to grow in the isocitrate medium, the tubes remaining clear as in the citrate medium. Seven of the *A. aerogenes* grew slowly in the isocitrate medium, whereas the remaining 13 failed to grow throughout an observation period of 10 days at 30 C. Evidently isocitrate under these conditions is utilized with more difficulty than is citrate and does not afford the same differentiation of the coli and aerogenes types as that given by citrate.

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VITAMIN DEFICIENCIES OF SEVEN STRAINS OF ECTOTHRIX, LARGE-SPORED TRICHOPHYTONS ISOLATED FROM MAN AND CATTLE

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In 1942, the senior author reported that a strain of *Streptomyces* (*Actynomyces*) *albus* stimulated the growth of a strain of *Trichophyton discoides* Sabouraud, common cause of tinea in calves and children in Uruguay, on a glucose peptone agar. He also found that the addition of thiamine to the medium had the same effect. The ability of *Streptomyces albus* to synthesize thiamine was proved by the growth of *Phycomyces blakesleeanus*.

Later on, Robbins, Mackinnon, and Ma (1942) reported that Mackinnon's strain of *Trichophyton discoides* had a complete deficiency for pyridoxine, DL-inositol, and molecular thiamine. In addition, there were partial deficiencies for unidentified substances present in peptone, casein hydrolyzate, hydrolyzed egg albumen, malt extract, gelatin, and a filtrate prepared from white potatoes. These partial deficiencies could not be satisfied by biotin, riboflavin, folic acid, pimelic acid, *para*-aminobenzoic acid, pantothenic acid, hypoxanthine, guanine, nicotinamide, 2-methyl-1-1.4-naphthoquinone diacetate, seven pyrimidine and purine bases, and 43 amino acids.

After our studies with strain 688, which we identified as *T. discoides*, we thought that vitamin deficiencies might be useful for the identification of species; that is, that strains of the same species would show the same deficiencies. Trying to elucidate this interesting problem we cultivated 5 strains of *T. discoides* and 2 strains of *T. ochraceum* on synthetic media with pyridoxine, thiamine, and DL-inositol, alone and in all possible combinations.

EXPERIMENTAL STUDY

Materials and methods. The organisms were cultivated at 30 C on agar slopes in neutral glass test tubes, 20 by 150 mm. Each tube contained 8 ml of a basal medium composed, per liter, of 50 g glucose, 2.0 g purified asparagine, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, and 15 g purified agar. To this medium 0.1 ml of the following solution of mineral supplements was added: 5.7 mg H_3BO_3, 18.6 mg $CuSO_4 \cdot 5H_2O$, 173 mg $(NH_4)_2Fe_2(SO_4)_4 \cdot 24H_2O$, 7.1 mg $MnCl_2 \cdot 4H_2O$, 3.6 mg ammonium molybdate (85 per cent), 79 mg $ZnSO_4 \cdot 7H_2O$, and 100 ml redistilled water.

This medium was autoclaved for 20 minutes at 115 C. The growth factors were added before solidification of the medium in the dose of 5 millimicromoles of thiamine, 5 millimicromoles of pyridoxine, and 1 mg of DL-inositol, singly and in all possible combinations. At the same time tubes were prepared containing the basal medium plus 0.1 g peptone (Difco) and 5 millimicromoles of thiamine. To another series of tubes nothing was added (basal medium).

Consequently we prepared 9 kinds of media (table 1). On the basal medium only a scant growth is possible, and on the basal medium with peptone and thiamine a luxuriant growth was observed. We indicated by (+++) the growth of the assayed strains on the optimal medium (with peptone and thiamine). We indicated by (−) all thin and scant growth like those observed on the basal medium (subcultures on the same medium are not possible). We indicated by (++) a great and remarkable increasing of the growth, and by (+) a little, although clear, improvement of the growth of the colonies. Inoculations were made by transferring, from a young culture on Sabouraud glucose agar, a small piece of mycelium about the size of a pinhead. All experiments were done in triplicate and repeated at least twice.

Cultures studied.

Strain 197. *Trichophyton discoides*. Sabouraud's strain.
Strain 688. *T. discoides*. Isolated from inflammatory ringworm of a man who used to industrialize cattle bristle.
Strain 457. *T. discoides*. Isolated from inflammatory tinea of the scalp (kerion) of a child who lived on a farm.
Strain 161. *T. discoides*. Isolated from ringworm of the face in a child who acquired the infection from calves.
Strain 1007. *T. discoides*. Isolated from inflammatory tinea of the arm in a child who used to caress calves.
Strain 461. *T. ochraceum*. Isolated from a calf with tinea of the head.
Strain 644. *T. ochraceum*. Isolated from inflammatory ringworm of the leg in a child in the country.

Discussion of the identity of the strains assayed. There exist very few studies about the ectothrix, large-spored (megasporic) trichophyton. After the basic works of Sabouraud (1908, 1910) the most important papers are those of Cazalbou (1914) and Lebasque (1934). Sabouraud described three species named *T. album*, *T. discoides*, and *T. ochraceum*. Other species were described later on the basis of minimal differences in the appearance of the giant colonies. The three classical species of Sabouraud have the same parasitological characteristics and the diagnosis must be based only on the appearance of the colonies on Sabouraud's glucose and maltose agar prepared with special peptones and special impure sugars. A revision of the methods is necessary. Nevertheless, the giant colonies of the three species have some common characteristics: they are glabrous or only a very short velvety aerial mycelium is produced; they are hard and elastic, and when we take a piece of the colony a little of the agar is also taken up; they produce filaments in the depth of the agar; and primary cultures are not easy to obtain (we frequently fail to obtain cultures from the lesions). Strains 197, 688, 457, 161, and 1007 may be identified, without doubt, as *T. discoides*. One of these strains is the type strain of Saboraud (strain 197). These strains never produced ochraceus colonies. On the other hand, strains 461 and 644 sometimes show a pale ochraceus pigment. We think that this is not a sufficient reason to classify these two strains in a different species, but most of the medical mycologists would identify them as *T. ochraceum*. We think also that *T. album* cannot

be surely distinguished from *T. discoides*. We think that *T. album, T. discoides,* and *T. ochraceum* are synonyms.

Conant and collaborators (1944) claim that *T. album, T. discoides,* and *T. ochraceum* are synonyms of *T. schönleini,* the agent of tinea favica. We think that this opinion is mistaken owing to the following reasons: (a) The parasitic form of *T. schönleini* is quite different from the parasitic form of the ectothrix,

TABLE 1

The results obtained with nine media

STRAIN	1 BASAL MEDIUM	2 BASAL MEDIUM + PEPTONE + THIAMINE	3 BASAL MEDIUM + THIAMINE	4 BASAL MEDIUM + PYRIDOXINE	5 BASAL MEDIUM + DL-INOSITOL	6 BASAL MEDIUM + THIAMINE + PYRIDOXINE	7 BASAL MEDIUM + THIAMINE + DL-INOSITOL	8 BASAL MEDIUM + PYRIDOXINE + DL-INOSITOL	9 BASAL MEDIUM + THIAMINE + PYRIDOXINE + DL-INOSITOL
197 T. discoides Sabouraud's strain...	−	+++	++	−	−	++	++	−	++
688 T. discoides.	−	+++	−	−	−	−	−	−	++
457 T. discoides.	−	+++	−	−	++	−	++	++	++
161 T. discoides.	−	+++	−	−	+	−	+	+	+
1007 T. discoides.	−	+++	−	−	++	−	++	++	++
461 T. ochraceum......	−	+++	−	−	−	−	++	−	++
644 T. ochraceum......	−	+++	−	−	++	−	++	++	++

megasporic trichophytons. (b) *T. schönleini* produces a noninflammatory tinea of man, whereas the megasporic, ectothrix trichophytons produce very inflammatory infections. (c) *T. album, T. discoides,* and *T. ochraceum* are genuine parasites of cattle and aberrant parasites of man, but *T. schönleini* is a genuine parasite of man. These arguments may be read in Saboraud's fundamental papers and were confirmed by Lebasque.

Results. Our results are recorded in table 1. *Trichophyton discoides,* strain

688, has complete deficiencies for thiamine, pyridoxine, and DL-inositol. Our results in 1945 and 1946 are identical with those of 1942. Strain 197 (Sabouraud's strain) has a complete deficiency only for thiamine. Strains 457, 161, and 1007 have a complete deficiency only for DL-inositol. *Trichophyton ochraceum*, strain 461, has a complete deficiency for both thiamine and DL-inositol. Strain 644 has a complete deficiency only for DL-inositol.

<h2 style="text-align:center">DISCUSSION</h2>

The nutritional requirements of the parasite molds have not been much studied. The authors always refer to the species and not to a single strain, although usually they study only one strain. We also expected to find all the strains of a species to have the same nutritional requirements. But among five strains of *T. discoides* we found at least three biological or nutritional varieties, types, or subspecies, which may be distinguished because their vitamin requirements are different: three strains have a complete deficiency for DL-inositol, one strain a complete deficiency for thiamine alone, and one strain a complete deficiency for DL-inositol, pyridoxine, and thiamine. The two strains of *T. ochraceum* are also different: one strain has a complete deficiency for DL-inositol, and the other a complete deficiency for DL-inositol and thiamine.

We may conclude also that the two strains named *T. ochraceum* have deficiencies similar to those of the strains named *T. discoides*. As we think that *T. discoides* and *T. ochraceum* are not different species, we have found four nutritional varieties among seven strains of the same species.

Vitamin deficiencies of a strain named *Trichophyton album* were studied by Schopfer and Blumer (1942, 1943). Only a partial deficiency for biotin was found. The synthesis of biotin was more rapid in a medium with asparagine than in a medium with ammonium sulfate. In a medium with ammonium citrate the synthesis of biotin was so slow that *T. album* seemed to be almost heterotrophic for biotin.

Schopfer (1944) wrote that in the same genus one species may be auxo-autotrophic and another species auxo-heterotrophic. But our results allow us to conclude that in the same species different strains may show different vitamin requirements. We must refer our results to the strain assayed and not to the species as a whole, until we have studied an appropriate number of strains.

The experiments of Beadle and Tatum on *Neurospora crassa* are interesting for us. Tatum and Beadle (1942) exposed *N. crassa* to X-rays and obtained a variant that was unable to synthesize *para*-aminobenzoic acid. They obtained also mutants that needed the addition of pyridoxine and of the thiazol of thiamine. Later on, Tatum (quoted by Wolff and Wolff, 1947) obtained mutants that required each of the vitamins of group B except folic acid and riboflavin. Other mutants required most of the amino acids. In our particular case, we think that losses in the ability to synthesize growth factors, and perhaps enzymes and amino acids, have produced a number of physiological races in a species that must be named *Trichophyton album* according to the law of priority.

Emmons and Hollaender exposed a suspension of spores of *Trichophyton*

mentagrophytes (1939*a,b*) to the action of monochromatic ultraviolet radiation. Besides the lethal effect, they observed the production of mutants which were classified in six groups. They observed that both mutant production and lethal effects are greatest when the spores are exposed to radiation of wave lengths 2,537 and 2,650 A. Hollaender and Emmons (1939*a,b*) speculated about the structure inside the cell which would be responsible for the sensitivity to ultraviolet radiation, and they wrote: "As in bacteria and yeast the wave lengths most toxic are 2,537 to 2,650 A—the wave lengths most highly absorbed by nucleic acids rather than by proteins."

We think that it is possible that the different aspects of Emmons and Hollaender's mutants may be due to changes in the nutritional requirements, perhaps to losses in the ability to synthesize growth factors.

SUMMARY

The effects of thiamine, pyridoxine, and DL-inositol, singly and in all possible combinations, on the growth of several strains of *Trichophyton discoides* Sabouraud and *Trichophyton ochraceum* Sabouraud have been studied on a synthetic basal medium with glucose and asparagine.

Among four strains of *T. discoides* isolated from man in Uruguay, three strains have complete deficiency only for DL-inositol, and one strain has complete deficiencies for DL-inositol, pyridoxine, and thiamine.

Sabouraud's strain of *T. discoides* has a complete deficiency only for thiamine.

Two strains of the species *T. ochraceum* have been studied. They were isolated in Uruguay. One strain, isolated from a calf, has complete deficiencies for DL-inositol and thiamine. The other strain, isolated from a child, has complete deficiency only for DL-inositol.

Consequently, the authors think that vitamin requirements cannot be useful for the identification of the species. It is also emphasized that the results of this kind of research must be attributed to the strain assayed and not to the species. Many nutritional varieties may be distinguished within a species.

The three ectothrix, large-spored (megasporic) trichophytons of Sabouraud named *T. album*, *T. discoides*, and *T. ochraceum*, may be considered as synonyms, but botanical, clinical, and epidemiological facts demonstrate that they are quite different from *Trichophyton* (*Achorion*) *schönleini*, the agent of tinea favica.

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FACTORS INFLUENCING THE PRODUCTION OF TETANUS TOXIN: GASEOUS PRODUCTS OF GROWTH

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Tetanus toxin of high potency (100 Lf per ml) is regularly produced in this laboratory (Mueller and Miller, 1947) by a variant strain of *Clostridium tetani*. Maximum yields require the presence of high concentrations of iron and appear to be sharply dependent on the size and type of container in which growth takes place. Titers of 100 Lf are attained in 6-by-⅝-inch test tubes containing 20 ml of medium. Flasks or bottles of various shapes and sizes are much less satisfactory, and no attempt at quantity production has resulted in yields of more than about 40 to 50 Lf and frequently less. It has recently been pointed out (Mueller and Miller, 1948) that the temperature of incubation influences the final titer in a relatively critical manner, and may offer a partial but by no means complete explanation for the phenomenon. It now appears that a more important factor is concerned with the free escape by diffusion of the gaseous products of growth and fermentation.

This was first observed in a series of attempts to measure gas evolved during growth and to correlate the measurements thus obtained with the final yields of toxin. It soon became evident that the mere presence of an inverted narrow tube in an ordinary test tube culture of the organism had an entirely unexpected effect on toxin production. Yields were irregular and in general far below those of control tubes. The most obvious difference in conditions was the fact that a certain amount of the gas produced was held in contact with the growing culture, whereas it normally diffused away promptly through the cotton plug. It was a simple matter to test this point, and the following experiment illustrates the effect.

Four tubes of ⅝-inch diameter each containing 20 ml of the usual medium employed for toxin production were so prepared as to vary the rapidity with which evolved gases could escape during incubation. The first two were 6 inches in length, whereas the second pair were 16 inches long. All four were covered with glass caps and sterilized in flowing steam for half an hour, cooled, and inoculated. Tubes 1 and 3 were then fitted with previously sterilized cotton plugs in the usual way. Tube 2 was closed with a 1-hole rubber stopper through which had been inserted a short length of glass tubing bent to an inverted **U** and drawn to a fine open capillary tip. Tube 4 was fitted with a 2-hole rubber stopper bearing a long glass tube which ended about 2 cm above the surface of the medium, for the entrance of sterile air, and a short exit tube. Incubation

was for 5 days at 35 C ± 0.1 in a Warburg bath.   During this period a very slow stream of sterile air was passed through tube 4.   The results follow:

Tube 1—usual cotton stopper (control)........................... 90 Lf
 "   2—glass capillary .... 20 "
 "   3—long; diffusion hindered by depth....................... 20 "
 "   4—long; ventilated....................................... 80 "

It seems reasonably clear that the only variable in this experiment must have been the composition of the gas in contact with the growing cultures.   This consists mainly of CO_2 along with some H_2 (Lerner and Pickett, 1945), but contains also appreciable amounts of H_2S or some similar volatile sulfide (odor and lead blackening).   Although proof is lacking, it seems probable that the sulfide is the unfavorable component of the mixture, although CO_2 may possibly also exert some influence.   It may be noted that the medium invariably blackens (FeS?) in the early stages of growth, and that the black, colloidal material flocculates and settles toward the end of the incubation period.   It is thus possible that a part of the role of the iron in the medium is to combine with sulfide.   This matter is being investigated further.

Recognition of the injurious effect of the retention of fermentative gases threw light on previously unexplained variations in toxin yields in earlier attempts at production in large containers.   Thus, it had been noted on several occasions that less toxin was obtained from cultures in long, narrow-necked flasks (volumetric type) filled well into the neck with broth than in Erlenmeyer flasks three-quarters full.   Such differences, of the order of 20 Lf in volumetric flasks as against 40 to 50 Lf in Erlenmeyers, had previously been completely unexpected, since it seemed probable that more favorable conditions for anaerobic growth would be obtained with a minimum exposure of surface to the air.

At present various expedients are being tried in order to provide more adequate disposal of gaseous products of growth in large containers.   It is hoped that these will lead to a practicable method adapted to large-scale production which can be reported later.

CONCLUSIONS

Maximal production of tetanus toxin appears possible only if the gases produced during growth can be largely and promptly dissipated from the culture.

This fact offers the most probable explanation yet available for the relatively poor yields of toxin in large containers.

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CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

VII. Effects on Activity of Alkaline Phosphatase[1]

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In penicillin-sensitive organisms the antibiotic blocks the normal functioning of sulfhydryl groups. The blocking results from —SH groups of essential polypeptides of the glutathione type being dehydrogenated more rapidly than they are rehydrogenated; exposure of the cells to bacteriostatic concentrations of penicillin results in "decompensated respiration," which is evidenced by a shift of sulfhydryl (—SH) to disulfide (S—S), of aldehydic COH to carboxylic COOH or ketonic CO, or of enolic COH to ketonic CO. Concomitantly with (or because of) these metabolic disturbances in *Staphylococcus aureus* there occur loss of gram-positiveness, loss of ability to absorb silver and other ions from the substrate, loss of capacity to stain with vital dyes such as neutral red, or with basic dyes in general, and loss of ability to retain a number of normal cell constituents such as amino acids, lipids, phospholipids, nucleic acid derivatives, etc. These observations have been reported in a series of publications (Dufrenoy and Pratt, 1947a,b, 1948; Pratt and Dufrenoy, 1947a,b, 1948a,b). Subsequently, Massart, Peters, and Van Houcke (1947) reported that penicillin at concentrations of $1:10^3$ to $1:10^4$ inhibits ribonuclease, and Macheboeuf (1948) observed that penicillin specifically inhibits mononucleotidase. The —SH⇌S—S relationship has been stressed in our earlier reports. The present paper is concerned primarily with the changes induced by penicillin in another reversible system normally operating in aerobic cells, i.e., aldehydic COH⇌carboxylic COOH and the correlative changes in the activity of alkaline phosphatase, the enzyme which "catalyzes the hydrolysis of simple esters of orthophosphoric acid with phenolic or alcoholic hydroxyl groups (Danielli, 1946)."

Penicillin at an optimal concentration may be considered as disturbing the normal equilibrium between ribo- and desoxyribonucleic acid fractions (Dufrenoy and Pratt, 1948). In this connection it is interesting to note that in a number of species of bacteria treatment with toluidine blue reveals the presence of metachromatic material, which may be associated with ribonucleic acid in the vacuole, and which apparently being closely related to adenosine triphosphoric acid (Wiame, 1947) plays a fundamental role in metabolism. This observation is significant, also, since it is known that microorganisms are most sensitive to penicillin when they are in stages of development that are characterized by a

high degree of basophily, which depends on the presence of ribonucleic acid (Wiame, 1947; Dustin, 1947), and that as they lose their basophilic character with age they become less susceptible to the action of the antibiotic.

MATERIALS AND METHODS

The test organisms used in this work were *Staphylococcus aureus* NRRL 313 and *Bacillus subtilis* NRRL B-558. Experiments were made with organisms grown in broth, on agar plates prepared and incubated according to the standard method prescribed by the FDA for cylinder plate assays of penicillin, and on plates prepared and incubated according to the modified technique previously reported (Dufrenoy and Pratt, 1947*a et seq.*). The penicillin that was used was a crystalline preparation of sodium benzyl penicillin that assayed 1,560 Oxford units per mg by the cylinder plate technique. The concentration of penicillin in the solutions that were used ranged from 0.25 to 4 units per ml. It has been estimated (Pratt and Dufrenoy, 1948*a*) that the concentration of penicillin at the margin of the inhibition zones formed around cylinders containing solutions of these concentrations is of the order of 0.001 to 0.01 unit per ml.

The sites of activity of alkaline phosphatase on assay plates were detected by flooding the plates with a saturated solution of triphenolphthalein phosphate,[s] incubating for 1 hour at 38 C, and developing the red color either by exposing to the fumes of ammonia or by alkalizing with 1 per cent aqueous solution of NaOH, following established procedures (Bray and King, 1942, 1943; Bray, 1944; Sols, 1947). For convenience, triphenolphthalein phosphate will henceforth be designated as TPP. Activity may be demonstrated in broth cultures by the addition of TPP, reincubation for 1 hour, and subsequent alkalization, whereupon a red color develops if active alkaline phosphatase is present.

EXPERIMENTS AND RESULTS

Detection of alkaline phosphatase and of phosphates. When standard assay plates seeded with *S. aureus*, which is known to be endowed with strong alkaline phosphatase activity, are subjected to the treatment described above, the zones of inhibition appear clear on a red background. The red color develops more rapidly and is more intense in the ring of enhanced growth that circumscribes each inhibition zone than in the general background. The ring of enhanced growth is thus revealed as the site of most intense alkaline phosphatase activity. This region is also richest in phosphorous compounds, as is demonstrated by the fact that it becomes black immediately following treatment with a 1 per cent aqueous solution of cobaltous nitrate followed by a 1 per cent aqueous solution of ammonium sulfide, whereas the remainder of the plate fails to react. In time the entire uninhibited background darkens somewhat, but the reaction never becomes as intense as in the ring of enhanced growth. This test depends upon linking the phosphate to cobalt and then converting the cobalt phosphate to cobalt sulfide, which is insoluble and black and is, therefore, readily visible.

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[s] Kindly prepared by Mr. Edward Alpen in the laboratory of Dr. W. D. Kumler in this college.

This corresponds to using the Gomori technique to study the redistribution of phosphates on the plate, irrespective of the cause of that redistribution. Localization of phosphates in the ring of enhanced growth can be demonstrated likewise by treating the plates with the molybdenic reagent, whereupon a blue color develops more intensely in this region than elsewhere on the plate (Pratt and Dufrenoy, 1947b).

It should be pointed out that in order to secure good growth of the test organisms it was necessary to provide phosphorous compounds in the substrate. Therefore, positive tests for phosphates were obtained in all parts of the seeded layer. It is noteworthy, however, that a notably higher concentration occurred in the ring of enhanced growth. This may be ascribed to the high metabolic activity of the cells in this region, which thus depleted the initially available supply of soluble phosphates, thereby creating a diffusion gradient so that phosphates were actually drawn into this area from other regions of the plate, especially the zone of inhibition where the low activity of the cells left a surplus available to be drawn into the region of higher phosphate demand. Moreover, under the effect of bacteriostatic concentrations of penicillin the microorganisms would release into the medium organic phosphorylated compounds which would then diffuse into the regions of intense growth and there be utilized as growth factors.

In order to study the distribution of alkaline phosphatase on agar plates containing phosphate we used the TPP reagent. Where phosphatases are active, phenolphthalein is liberated and yields a red color upon alkalization. Thus interference from phosphates, either pre-existing in the medium or enzymatically liberated, is avoided. The reagent can be applied in such a way as to provide a pattern of color intensities representative of the distribution of enzymatic activity.

It should be pointed out that the region shown to possess high phosphatase activity is also the site of greatest dehydrogenase activity, as revealed by the triphenyltetrazolium chloride reagent (Dufrenoy and Pratt, 1948), as well as the site of maximal response for reactive sulfhydryl and aldehydic groups (Dufrenoy and Pratt, 1947a; Pratt and Dufrenoy, 1947b, 1948b). Dehydrogenase activity is greatest on 3-hour plates,[4] although it is also readily demonstrable on older plates, including those that have been incubated 16 to 18 hours. Phosphatase activity, on the other hand, although it is readily demonstrable on 16- to 18-hour plates, where autolysis is already prominent, is only barely perceptible on 3-hour plates. This is in agreement with the observation of Roche et al. (1945) that the activity of phosphatase may depend upon the presence of activators that are liberated during proteolysis. The activity of this enzyme system may, therefore, be expected to be affected by lysis, either occurring naturally or induced by appropriate concentrations of penicillin. This emphasizes the importance

[4] By "3-hour plates" we mean plates that have been exposed to penicillin for only 3 hours during a secondary incubation period that follows a primary incubation period without penicillin, the object of which is to permit the cells to reach the logarithmic phase of growth in which they are most reactive to penicillin.

of the physiological age of the test organisms in studies concerned with the evaluation of different reagents or of different cell systems.

Standard plates (i.e., plates with 16 to 18 hours of incubation with penicillin) seeded with *B. subtilis* give a strong positive test for dehydrogenase activity but fail to give a positive response for phosphatase. This is in accord with the observation of Bayliss, Glick, and Siem (1948), who failed to obtain a positive test for phosphatase in cells of *B. subtilis* unless the enzyme system was activated by the addition of alanine plus manganese or of alanine plus magnesium to the substrate. Pett and Wynne (1938) have also shown that there are marked variations in the phosphatase activity of different strains of *B. subtilis* and that the phosphatase activity depends upon the composition of the substrate. A threshold that delimits the inhibition zones from the uninhibited background may be demonstrated on *S. aureus* plates treated with appropriate reagents for reducing groups, for dehydrogenases, or for alkaline phosphatase. In serial dilution studies in broth, a similar threshold that partitions tubes that give no response from those that give a positive color reaction has been demonstrated with the same reagents. Experimental results show that the threshold for activity of alkaline phosphatase and for dehydrogenase obtains at about 0.01 unit per ml, which corresponds to the concentration of penicillin previously estimated to occur at the margins of inhibition zones on assay plates (Pratt and Dufrenoy, 1948a). The principle that is involved in the rapid assays on plates (Goyan, Dufrenoy, Strait, and Pratt, 1947; Pratt and Dufrenoy, 1947c; Pratt, Goyan, Dufrenoy, and Strait, 1948) applies equally well to assays by the serial dilution method. Either the triphenyltetrazolium chloride reagent for dehydrogenases or the TPP reagent for alkaline phosphatase can be used to define the threshold between bacteriostatic and subbacteriostatic concentrations of penicillin after a much shorter period of time than is required for estimation of the end point by means of turbidimetry.

Detection of aldehydic groups on assay plates. Since the threshold for alkaline phosphatase activity on assay plates (see above) coincides with the previously demonstrated threshold for —SH groups, i.e., both are lacking in the zone of inhibition and are prominent in the circumjacent ring of enhanced growth, and since there may be correlation between the rate of hydrolysis of phosphorylated compounds and the oxidation-reduction potential (Roche *et al.*, 1945, Morel *et al.*, 1947) it appeared worth while to attempt to obtain direct evidence of the presence of aldehydic groups at those sites. To this end, plates seeded with *S. aureus* were carefully flooded with a solution prepared by dissolving 25 mg azo-benzene-phenyl-hydrazine-sulfonic acid[5] in 100 ml of 10 N H_2SO_4. This reagent is stated by the British Drug Houses, Ltd., to yield a ruby-red color with aliphatic aldehydes and a blue color with aromatic aldehydes. In our experiments, a purple color appeared most rapidly in the ring around each zone of inhibition and then

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[5] This compound is available from the British Drug Houses, Ltd., London, England. The solution of this reagent can be used to develop 3-hour plates, according to previously described methods (Dufrenoy and Pratt, 1947a; Pratt and Dufrenoy, 1947b, c), the zones of inhibition appearing as unstained areas sharply delimited by the purple background.

gradually spread throughout the uninhibited background. This confirms our earlier observation (based on several tests, including the Schiff reagent) that aldehydes, probably a mixture of aromatic and aliphatic, are present in abundance in the ring of enhanced growth but are absent, or at least are not demonstrable, with the tests used, in the zones of inhibition.

According to Genevois (1947) the following test is specific for aldehydes. A solution consisting of 1 volume of 10 per cent nitromethane in 55 per cent alcohol and 4 volumes of 5 per cent aqueous potassium carbonate is allowed to act on the test material at 40 C for 3 hours. Since this reagent is reported to block aldehydic groups, the failure of color to develop on test plates so treated and then exposed to the Schiff or any other suitable aldehyde reagent that gives a positive test on untreated plates could be taken as evidence of the involvement of aldehyde groups.

In order to ascertain whether the groups responsible for the positive tests that were observed with the Schiff reagent and with the azo-benzene-phenyl hydrazine sulfonic acid reagent were actually aldehydic, we pretreated assay plates with the solution proposed by Genevois before addition of the aldehyde reagent. We have observed that pretreatment with Genevois' solution completely prevents formation of color on the plates

DISCUSSION

It has been reported that "penicillin, in concentrations sufficient to arrest completely the growth of certain bacteria, was found to have no significant effect on the phosphatase activities of these bacteria (Bayliss, Glick, and Siem, 1948)." Using the TPP reagent, we have confirmed the observation that high concentrations of penicillin (100 units per ml) acting for a few hours do not inactivate alkaline phosphatase in *S. aureus*. This result is not at all surprising, since phosphatase activity is known to be independent of the survival of the cell. This is evidenced by the fact that such activity can be demonstrated in cells that have been fixed in acetone or other suitable agents (Danielli, 1946; Bayliss, Glick, and Siem, 1948).

In our work we have been concerned with concentrations of the order of those that one might expect to encounter in therapeutic practice. Our experiments show that in such concentrations penicillin inhibits the activity of alkaline phosphatases, as may be evidenced by use of the TPP reagent. The report of Natarajan and De (1946) supports our conclusions. They studied the effect of solutions containing from 0.25 to 1 unit per ml on the activity of bone phosphatase and observed that "penicillin in the maximal concentrations that can be produced in the tissues does not significantly inhibit bone phosphatase. On the other hand, a marked inhibiting action is noticed with lower concentrations of penicillin." Their data, covering the range from 0 to 1 unit per ml, afford another example of the typical maximal effect at an optimal dosage within the range of practical therapeutic concentrations. It is in this range that penicillin most effectively mediates a shift toward the right in the equilibrium —SH $\rightleftarrows$ S—S in the chain of events operating in the aerobic respiratory system; correlatively,

this entails a shift from aldehydic COH to ketonic CO and to carboxylic COOH, as evidenced by the formation of fatty acids and the lowering of pH.

SUMMARY

A study was made of the distribution of phosphates, of aldehydes, and of alkaline phosphatase activity on different parts of penicillin assay plates seeded with *Staphylococcus aureus* or with *Bacillus subtilis.*

The molybdenic reagent and a modified Gomori test were used to demonstrate the location of phosphates. Aldehydes were revealed by the use of the Schiff reagent and by the use of a solution of azo-benzene-phenyl-hydrazine-sulfonic acid in H_2SO_4. Pretreatment of the plates with a solution containing nitromethane in alcohol and aqueous potassium carbonate, which Genevois has shown blocks aldehydic groups, prevented development of a positive test with the aldehyde reagents. This is taken as evidence that the positive tests obtained in the absence of pretreatment with the blocking agent were actually due to the presence of aldehydes. Sites of phosphatase activity were located by the use of triphenolphthalein phosphate followed by alkalization.

Positive reactions for phosphates, for aldehydes, and for alkaline phosphatase activity were obtained throughout the uninhibited regions, and were most intense at the sites of the ring of enhanced growth which surrounds each inhibition zone and which corresponds to a region in which the cells are exposed to appreciable but subbacteriostatic concentrations of penicillin. Only negative tests were obtained within the inhibition zones.

Serial dilution studies with broth cultures confirmed the results obtained on assay plates.

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THE UTILIZATION OF CARBON COMPOUNDS BY SOME ACTINOMYCETALES AS AN AID FOR SPECIES DETERMINATION

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Attention has recently been focused on the identification of the actinomycetes because of the ability of some members of this group to produce antibiotics. The determinative procedures now in use are based primarily on morphology, pigmentation, and growth characteristics on media composed of complex natural substrates. With such media one can often observe a variety of pigments and colony types produced by the same organism. In inexperienced hands, this leads to difficulties in interpretation of results, and one is often forced to designate a particular isolate as one of several species rather than one distinct species. To aid in the identification of a group of antibiotic-producing actinomycetes under study, an intensive investigation of their utilization of carbon compounds in chemically defined media was undertaken.

Prior to the current investigations a number of workers had studied the utilization of carbon compounds by some species in this group. Beijerinck (1900), Sames (1900), Caminiti (1913), Fousek (cited by Waksman, 1919), Krainsky (1914), and Gray and Thornton (1928) each tested a few materials and found that various members of this group could grow on media containing different carbon sources. More extensive tests on carbon compounds were made by Salzmann (1907), Munter (1913), and Waksman (1919). Recently Taylor and Decker (1947) showed that various plant pathogenic actinomycetes could be separated from nonpathogenic isolates on the basis of their reactions on carbon compounds, as well as other criteria. The studies of Cochrane (1947) also indicated that different species in the genus *Actinomyces* might be separated on the basis of acid production.

METHODS

Routine identifications. Gram reactions were determined, condidial and hyphal dimensions were measured, and the growth characteristics and responses of the various isolates were observed on the following media; nitrate broth, tryptone broth, starch agar, Kligler's double sugar medium, gelatin, litmus milk, tyrosine broth, nutrient broth, D-glucose agar, Czapek's sucrose agar, calcium n butyrate agar, Dorset's egg medium, Loeffler's serum medium, potato slants, and carrot slants. Insofar as possible, all media prepared and procedures followed were those recommended in *A Manual of Methods for Pure Culture Study of Bacteria* (1946), or cited in *Bergey's Manual of Determinative Bacteriology* (1939, 1946). Some of the results thus obtained were used in species identification of each of the

isolates, following the key in *Bergey's Manual* (1946).[1] The identities of the different acquisitions are recorded in table 1 and some of the more important characteristics in table 2.

Reaction on chemically defined media. Stock cultures of the isolates were maintained on a synthetic medium containing either D-glucose or starch as a carbon source, or in nutrient broth (tryptone 0.5 per cent, yeast extract 0.3 per cent). The utilization of carbon compounds was tested on the following basal medium:

$(NH_4)_2SO_4$	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4 \cdot 7H_2O$	1.00 g
$CuSO_4 \cdot 5H_2O$	0.0064 g
$FeSO_4 \cdot 7H_2O$	0.0011 g
$MnCl_2 \cdot 4H_2O$	0.0079 g
$ZnSO_4 \cdot 7H_2O$	0.0015 g
Difco agar	15.00 g
Distilled water	1,000 ml

This medium was adjusted to pH 6.8 to 7.0, tubed in $9\frac{1}{2}$-ml amounts, and autoclaved. After cooling to about 45 C, sterile aqueous solutions of the carbon compounds were added to give the proper concentration. The carbohydrates, polyhydric alcohols, DL-inositol, and salicin were added such that that the final concentration was 1 per cent, the phenols 0.1 per cent, and the sodium salts of the organic acids 0.15 per cent. Those materials sufficiently soluble in water were sterilized by filtration through Seitz EK filter pads. Some compounds (dextrin, starch, dulcitol, DL-inositol, and salicin) that were relatively insoluble or did not filter well were added directly to the basal medium in the proper concentration prior to tubing and sterilization. After addition of the carbon sources, the tubes were slanted, allowed to solidify, and incubated to determine sterility.

Inocula were prepared by growing the isolates in nutrient broth (Difco tryptone 0.5 per cent, yeast extract 0.3 per cent) at room temperature for 2 weeks. The liquid was decanted, and the remaining mycelium was washed with sterile distilled water, transferred to 100 ml of sterile distilled water, and thoroughly shaken. One-half ml of this suspension was allowed to flow over the surface of the test medium. Controls consisting of tubes of basal medium alone were always inoculated with each run. Observations for the presence or absence of growth were made after 10 days' incubation at 26 C. The ability of the different compounds to support growth was tested in 2 to 14 replicate tubes for each material and the experiments were usually repeated from 2 to 9 times during an interval of 18 months.

<center>RESULTS</center>

Identification of isolates. The characteristics of those cultures that were received under specific names agreed with our determinations except for minor

differences (tables 1 and 2). The unknown isolates, however, were harder to identify because of difficulties in the evaluation of pigment production and colony types. Some of the characteristics so determined could not be appropriately

TABLE 1

Source and identity of cultures

ISOLATE	SOURCE	IDENTITY
Streptomyces griseus A2.... ...	S. A. Waksman, Rutgers Univ.	As received
" " B..	G. H. Savage, The Upjohn Co.	" "
" " 72........	H. W. Anderson, Univ. of Ill.	" "
" " 434........	A. J. Whiffen, The Upjohn Co.	" "
S. lavendulae 3440-14...	S. A. Waksman, Rutgers Univ.	" "
A. "	ATCC	" "
A-56, probable strain of *S. lavendulae*....	A. J. Whiffen, The Upjohn Co.	*S. lavendulae*
A-151 probable strain of *S. lavendulae*	" " " " " "	" "
A-10 variant of *S. lavendulae*....	H. W. Morton, Univ. of Penn.	" "
S. antibioticus	S. A. Waksman, Rutgers Univ.	*S. antibioticus*
A. scabies.	W. D. Thomas, Col. State Coll.	As received
A. scabies 3352	ATCC	" "
A. albus 618............ ...	ATCC	" "
A-105, *A. erythreus, fradiae, albosporeus,* or *californicus*.......	H. E. Morton, Univ. of Penn.	*S. fradiae,* or *albosporeus*
83D, unidentified................	H. W. Anderson, Univ. of Ill.	*S. flavovirens*
211, unidentified.	C. H. Meredith, Iowa State Coll.	*S. erythrochromogenes*
8-48, unidentified................	D. Gottlieb, Univ. of Ill.	*S. griseolus, rutgernensis,* or *halstedii*
8-12, unidentified...............	" " " " "	*S. fradiae* or *albosporeus*
8-80, unidentified......... .. .	" " " " "	*S. fradiae, alboflavus,* or *californicus*
8-96, unidentified..	" " " " "	*S. fradiae, alboflavus,* or *californicus*
8-44, unidentified...	" " " " "	*S. lavendulae,* or *reticuli*
S. violaceous-ruber 3030..........	S. A. Waksman, Rutgers Univ.	As received
Nocardia gardneri 3449...........	" " " " "	" "
N. asteroides 3308.....	" " " " "	" "
N. polychromogenes 3409..........	" " " " "	" "
Micromonospora sp. 3450.........	" " " " "	*M. fusca*
" " 3451........	" " " " "	" "

fitted into the key. For this reason some of the isolates were given more than one possible species designation (table 1).

Growth responses to carbon sources in chemically defined media. Generally, the

TABLE 2

Cultural characteristics of various actinomycetes* · †

ISOLATE	H₂S	GELA-TIN LIQ.	TYRO-SINASE	CZAPEK'S MEDIUM	DORSET'S EGG MEDIUM	LOEFFLER'S SERUM	STARCH MEDIUM	POTATO
S. griseus (all strains)	−	+	−	P, A, wh to c	G, fl, gr- wh to brn	G, fl, gr- wh to brn	G, A, gr- wh	G, A, wh
S. lavendulae (all strains)	+	+	+	F, A, lav to wh	F, S, dk brn	F, Sm, dk brn	G, A, lav- wh	G, A, lav
S. antibioticus							G, Sm, brn	
A. scabies	−	+	−	G, A, wh	G, A, wh	G, A, wh	G, A, wh	G, A, wh
A. scabies 3352	+		+	P, A, c	G, Sm, gr- wh	G, S, gr- wh	G, A, Sm, gr	G, A, wh
A. albus 618	−		−		G, Sm, wh	G, Sm, wh	G, A, Sm, wh	
S. flavovirens 83D9	+	+	+	F, A, wh-gr	G, Sm, yel	G, Sm, yel-gre	G, A, gr- wh	G, A, gr- gre
S. erythro-chromogenes 211	+	+	+	F, A, wh	G, Sm, rd-lav	G, Sm, gr-wh	G, A, Sm, lav- wh	G, A, wh-rd
A-105	−			F, A, Sm, lav- wh rd rev	G, A, Sm, rd- wh	G, A, Sm, wh- rd	G, A, lav-wh	G, A, Sm, wh- rd
S. violaceous-ruber 3030	−		+	G, A, Sm, wh- bl	G, Sm, wh	G, Sm, wh	G, A, Sm, wh- rd	G, A, wh
8-12	−	+	−	G, A, wh- lav or rev	G, A, wh	G, A, wh	G, A, Sm, wh- lav	G, A, wh rev rd
8-80 and 8-96	+	+	−	G, A, lav- wh to or- yel red rev	G, A, wh-yel	G, A, wh to yel	G, A, lav-wh to or-yel rev rd	G, A, wh-gr-rd rev or-rd
8-48	−	+	+	G, A, gr to wh	G, A, gr to wh	G, A, wh to gr	G, A, wh to gr	G, A, gr to wh
8-44	+	+	+	G, A, Sm, lav	G, A, wh to gr	G, Sm, dk brn	G, A, lav-wh	G, A, gr
N. asteroides 3308	−	−	−		G, Sm, yel or, rd	G, Sm, yel, rd, or	G, Sm, p, or	F, Sm, e or
N. poly-chromogenes 3409	−	−	−		G, Sm, p, yel, or rd	G, Sm, or yel, rd	F, Sm, p, or, rd, wh	F, Sm, p, wh-rd
N. gardneri 3449	−	+	−	F, Sm, wh	G, Sm, gr-gre	G, Sm, gre-gr	G, Sm, wh	F, Sm, wh
M. fusca 3450 and 3451	−	+	−	F, Sm, p, wh	G, Sm, p, or to blk	G, Sm, p, or to blk	F, Sm, p, or to blk	F, Sm, yel-brn

* All cultures reduced nitrates; none produced indole, and only one culture, *S. flavovirens*, liquefied Dorset's medium.

† Growth: G—good, F—fair, P—poor, A—aerial hyphae, Sm—smooth, p—punctiform, fl—flat.

Pigment: wh—white, gr—gray, lav—lavender, brn—brown, or—orange, blk—black, bl—blue, yel—yellow, rd—red, gre—green, c—colorless, rev—reverse of colony.

growth responses on the different media were well defined and were similar in the various experiments performed over a period of 18 months. The ability of the different isolates to grow on the test media varied considerably in different species (table 3). In every case, the control tubes showed very little or no growth, and those tubes in which the isolates could effectively utilize the particular carbon source had very profuse growth. Occasionally, only very slight growth was observed with some materials, indicating that the particular compound was not an adequate source of carbon in that concentration, or that the materials used contained traces of other compounds. It is significant that all the species tested could be differentiated from one another on the basis of their growth on the various carbon sources alone (table 3) and these differences could be substantiated by other physiological characteristics (table 2).

The four strains of *S. griseus*, although obtained from different sources, gave identical reactions, thus indicating a marked uniformity in the different isolates of this species. Similarly the two known strains of *S. lavendulae* (*Actinomyces lavendulae* and 3440-14) and isolate A-151, identified as *S. lavendulae*, likewise gave identical reactions with all but one of the compounds, sodium acetate. Of the remaining isolates tentatively identified as *S. lavendulae*, A-10 and A-56 differed from the accepted strains of *S. lavendulae* in the utilization of only two of the compounds.

Particular interest was placed on the results obtained with isolates 83D and 8-44. Isolate 83D produces actinomycin,[2] an antibiotic elaborated by *S. antibioticus* and other *Streptomyces* sp. (Waksman and Woodruff, 1940; Waksman, Geiger, and Reynolds, 1946). This isolate, which was identified as *S. flavovirens*, differs from *S. antibioticus* on six of the carbon sources tested as well as on the routine media; thus two different known species produce the same antibiotic. Isolate 8-44 resembles *S. lavendulae* or *S. reticuli* when determined by the usual identification procedures, but appears quite different from *S. lavendulae* in its ability to utilize some pentoses and sodium acetate. In addition, 8-44 produces only the antibiotic, chloromycetin, whereas *S. lavendulae* produces streptothricin.

Isolate A-105 resembles to a large degree isolates 8-12, 8-80, and 8-96, by the usual keys, but differs from them in the utilization of four of the compounds tested. Both isolates A-105 and the 8-80 group[3] produce antibiotics with some similar properties, but these antibiotics have not been completely characterized.

The two strains of *S. scabies*, which appeared different when the routine procedures for identification were used, also exhibited a number of differences in ability to utilize the various carbon sources tested. Twelve differences on the test media combined with the differences observed during preliminary identification might indicate that one of these isolates is not a true *S. scabies*, or that strain differences in this species are very marked.

With the exception of *N. gardneri*, the species of *Nocardia* and *Micromonospora*

TABLE 3

Growth of test organisms on synthetic medium plus various carbon sources*,†

COMPOUND	S. griseus 72	A2	B	434	S. lavendulae A. lav.	3440-14	A-151	A-56	A-10	ISOLATE 8-44	8-80	8-96	8-12	A-105	S. antibioticus	S. flavovirens 83D	ISOLATE 8-48	211	Nocardia gardneri 3449	S. scabies A. scabies	3352	S. albus 618	S. violaceousruber 3030
L-Xylose	+	+	+	+	−	~	(−)	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+
L-Arabinose	(−)	+	(−)	(−)	−	~	(−)	−	−	+	+	+	+	+	+	+	+	+	+	(−)	(−)	+	+
Rhamnose	+	−	+	+	−	−	(−)	−	−	+	+	+	+	+	+	+	−	+	+	+	+	−	+
D-Fructose	+	+	+	+	~	+	+	+	(−)	+	+	+	+	+	+	+	~	+	+	+	+	+	+
D-Galactose	+	+	~	~	+	−	+	+	(−)	+	+	+	+	+	+	+	(−)	+	+	+	+	~	~
Sucrose	~	~	+	+	−	+	−	−	+	−	+	+	+	+	(−)	+	+	+	+	+	−	+	+
Maltose	+	+	+	+	+	+	+	+	+	~	+	+	+	+	(−)	+	(−)	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	(−)	+	+	+	−	−	−
Inulin	+	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	+	+	+	+	+	+	+	(−)	(−)	(−)	(−)	−	(−)	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	(−)	+	+	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	(−)	(−)	(−)	(−)	(−)	−	(−)	(−)	(−)	+	+	+	+	+	(−)	(−)	+	(−)	−	−	−	+	+
Dulcitol	−	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	−	+	+
DL-Inositol	+	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	+	+	(−)	+	+	(−)	(−)	(−)	(−)	−	−	−	(−)	(−)
Salicin	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	+	+	+	+	+	+	+	+	+	+	(−)	−	+	+
Na-acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(−)	+	+
Na-citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	~	+	+	+	(−)	+	~
Na-succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(−)	(−)	+	(−)	−	+	(−)

* All isolates utilized D-glucose, D-mannose, cellobiose, starch, dextrin, and glycerol; none of the isolates utilized phenol, o-cresol, m-cresol, sodium formate, oxalate, tartrate, or salicylate.

† Key: + = growth and positive utilization; − = no growth, no utilization; (−) = faint growth, probably no utilization; ? = variable reaction, growth positive at times, negative at others.

gave inconclusive results, since these microorganisms did not grow well on the test media.

Using the information provided in table 3, a flow sheet (figure 1) was prepared on which all the antibiotic-producing isolates could be separated on the basis of their growth responses. Other simple tests could be readily fitted into this

```
Rhamnose −                      Rhamnose +         Rhamnose +
Raffinose −                     Raffinose +        Raffinose −
    |                               |                  |
  S. griseus                    8-80 group          8-44
  S. lavendulae                 S. flavovirens      S. antibioticus
    |                           N. gardneri
                                A-105

Xylose −        Xylose +
Mannitol −      Mannitol +
Lactose −       Lactose +
Na-acetate (−)  Na-acetate +
    |               |
(S. lavendulae)  (S. griseus)


Dulcitol −      Dulcitol +      Dulcitol −
Inositol +      Inositol +      Inositol −
    |               |               |
8-80 group      (A-105)         (N. gardneri)
S. flavovirens


Na-acetate +    Na-acetate −
Na-succinate −  Na-succinate +
    |               |
(8-80 group)    (S. flavovirens)


                                Mannitol +          Mannitol −
                                Inositol +          Inositol −
                                    |                   |
                                (S. antibioticus)     (8-44)
```

Figure 1. Flow sheet for the separation of antibiotic-producing isolates on the basis of utilization of carbon compounds in a chemically defined medium.

scheme (table 2). Further to substantiate the differences between any two species on the flow sheet other responses from among those listed in table 3 could be chosen. A knowledge of the utilization of carbon compounds in a chemically defined media should be a valuable addition to determinative keys and aid in the identification of members of the *Actinomycetales*, especially the genus *Streptomyces*. It is of special interest when a small group of organisms are concerned

such as the antibiotic-producing actinomycetes; among these most of the species can be separated by such reactions alone.

SUMMARY

Twenty-seven isolates of organisms belonging to the genera *Streptomyces,* *Nocardia,* and *Micromonospora* have been tested for their ability to utilize 33 different carbon compounds as a source of carbon in a chemically defined medium. The results obtained with the antibiotic-producing streptomycetes and others indicate that such reactions can aid species identification. All of the streptomycetes studied were found to utilize D-glucose, D-mannose, starch, dextrin, and glycerol, but not erythritol, phenol, o-cresol, m-cresol, p-cresol, Na-formate, Na-oxalate, and Na-tartrate. Reactions on the other carbon compounds varied with the particular species.

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GROWTH AND ASSIMILATION IN CULTURES OF SACCHAROMYCES CEREVISIAE[1]

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Studies on microbial assimilations have shown that definite quantitative relationships exist between the amount of a foodstuff dissimilated and the amount assimilated by nonproliferating cells of a given species (see review by Clifton, 1946). Clifton and Logan (1939) observed approximately the same quantitative relationships between the extent of oxidation and assimilation in actively proliferating cultures as in washed suspensions of *Escherichia coli*. Whelton and Doudoroff (1945) observed that assimilation from a variety of substrates in cultures of *Pseudomonas saccharophila* was generally less than in suspensions, synthesis during growth in a glucose medium most closely approaching that observed with resting cells plus glucose. Since considerable work has been done on assimilation by yeasts, this study was initiated in an effort to compare the extent of assimilation in cultures with that observed in suspensions of *Saccharomyces cerevisiae*. Fink, Krebs, and Lechner (1939) observed in macro growth experiments that 90 per cent of the carbon postulated to be assimilated by washed suspensions according to the equation

$$C_6H_{12}O_6 \rightarrow 2CH_3CHO + 2CO_2 + 4H$$

was actually assimilated during the growth of *Torula utilis*. In the following studies on assimilation in cultures of *S. cerevisiae*, the problem of assimilation is more complex since marked changes in respiratory activity were noted during growth. Typical data will be presented and an attempt will be made to correlate the observations on assimilation in actively growing cultures with those previously reported for resting suspensions (Pickett and Clifton, 1943; Winzler, 1940).

EXPERIMENTAL PROCEDURE

One culture of *S. cerevisiae* freshly isolated from a cake of Fleischman's yeast and two laboratory strains were employed. These organisms were cultured in a basal medium adjusted to pH 6.6 to retard the marked shift in pH to as low as pH 2.5 during growth. Control experiments with media of pH 5.0 indicated that pH effects were not responsible for the growth and respiration results reported herein. The medium consisted of NH_4Cl, 2.0 g; K_2HPO_4, 1.0 g; $MgSO_4$, 0.5 g;

KCL, 0.5 g; and, in the experiments recorded here, 0.01 g $FeSO_4$. Trace salts (Snell et al., 1940, 1.0 ml to 1,000 ml of medium) were employed in some of the later experiments but had no influence on the results observed.   In a number of experiments a vitamin mixture was added to the basal medium to give the following concentrations in micrograms per liter: 100 B_1, 50 B_2, 50 B_6, 200 pantothenic acid, 50 para-aminobenzoic acid, 200 niacinamide, 400 inositol, 100 choline, 5 biotin, and a trace of folic acid.   Doubling the concentration of growth factors had no appreciable effect on growth or respiration.   Glucose, or other sugars, were added to give a concentration of 1.0 per cent.

The cultures were incubated in a constant temperature room at 25 C and were continuously aerated in a closed system similar to that described by Wood et al. (1940).   Carbon dioxide was determined by titration of residual potassium hydroxide in an adsorption vessel rather than by gravimetric methods.   Erlenmeyer flasks were employed as culture vessels, their contents being stirred with a magnetically driven stirrer.   Air was continuously circulated over the surface of the medium.   The inoculum in all experiments recorded here was from 24-hour cultures in the basal glucose medium enriched with vitamins with the exception of the experiment reported in table 1, in which the inoculum was from Sabouraud agar.

Glucose was determined by oxidation with ceric sulfate, alcohol by dichromate reduction, and carbon by the method of Van Slyke and Folch (1940).   Samples from the cultures were centrifuged in centrifuge combustion tubes, and as much as possible of the supernatant fluid was removed without disturbing the cells. The cells were then washed with acidified water, centrifuged from the suspension, and a layer of freshly precipitated barium sulfate was deposited over the cells by centrifugation.   This layer of barium sulfate greatly facilitated the removal of the supernatant wash fluid without disturbing the cells.   Numbers of yeast cells were determined by direct counting in a Petroff-Hauser counting chamber. Slight modifications in experimental procedure will be mentioned under individual experiments.

EXPERIMENTAL RESULTS

In a series of preliminary experiments it was observed that little or no oxygen was consumed in the first 16 to 24 hours of incubation of aerated cultures in the basal medium plus glucose.   Considerable multiplication and carbon dioxide production were noted during this time interval and analyses for ethyl alcohol indicated marked aerobic fermentation.   A much greater crop yield was obtained in 24 hours in nonaerated cultures, which suggested that oxygen under ordinary atmospheric tension might be inimical to the growth of yeast in the early growth phases.   Control-aerated growth experiments without carbon dioxide absorption indicated that the removal of carbon dioxide from the circulating air was not the factor responsible for the retardation of growth in aerated cultures.   Also growth experiments in Warburg flasks, with and without carbon dioxide absorption, gave results similar to those obtained in the Brewer-Werkman apparatus, thus ruling out the possibility of experimental error with this latter

apparatus.   On the assumption that a high oxidation-reduction potential might be inhibitory to oxygen consumption or enzyme formation, reducing agents such as thioglycolate, inorganic sulfides, thiosulfate, and cysteine were added to the basal medium with little or no change in the observed growth and metabolic acitivity.   In other experiments the growth factors and trace salts listed under experimental procedure were added separately and together to the basal medium plus glucose with little noticeable difference in metabolic behavior of the yeast as compared with that in the basal medium plus glucose control aerated cultures. The rate of growth was somewhat greater in the basal medium enriched with the vitamin mixture.   Therefore only the results of growth metabolism studies in the enriched basal medium will be presented here, as they are typical of those observed in the different experiments.   In the particular experiment reported in table 1, alcohol determinations are reported for alcohol in the medium and alcohol lost by the medium but absorbed by potassium hydroxide, the carbon dioxide

TABLE 1

Carbon utilization during growth in vitamin glucose medium

HOURS	ML MEDIUM	CELLS/ ML × 10⁶	ML O₂ CON- SUMED	ML CO₂ PRO- DUCED	R.Q.	MG C IN END PRODUCTS				MG GLUCOSE PER ML	MG C PER ML
						CO₂	Alcohol		Cells/ ml		
							Abs. in KOH	In me- dium			
0	60	1.67	—	—	—	—	—	—	0.01	10.50	4.21
16	60	24.5	0	67.1	∞	35.94	7.1	93.0	0.30	2.48	2.36
23	50	32.0	14.9	47.4	3.18	25.38	9.7	73.5	0.38	0.26	1.66
27	45	46.5	10.5	10.2	0.97	5.46	8.3	53.7	0.43	0.24	1.23
40	40	60.6	36.8	26.3	0.71	14.10	9.4	14.4	0.61	0.15	0.52
52	35	69.8	29.8	20.1	0.67	10.74	1.6	1.9	0.68	0.16	0.24
64	26	67.6*	8.3	8.4	1.01	4.50	0.1	0.5	0.67	0.17	0.20
72	20	64.0*	3.5	2.9	0.83	1.56	0	0	0.65	0.16	0.18

* Remnants of disintegrating cells seen, only intact cells counted.

absorbent.   Some error may be introduced by a small amount of nonabsorbed alcohol in the circulating air stream.   Supernatant carbon values are low, because of the loss of alcohol by volatilization on the addition of combustion fluid.   In subsequent experiments the combustion flasks were held in ice water during the addition of combustion fluid, which was added dropwise, and carbon balances were much closer to 100 per cent.   Carbon balances, calculated on the basis of the original 60 ml of medium, are recorded in table 2.

In another experiment the behavior of two laboratory strains of *S. cerevisiae* was compared with that of the strain freshly isolated from Fleischmann yeast. There was no appreciable difference in growth and metabolic activity of the three strains with the exception that one strain (FF 17, obtained from the American Type Culture Collection in 1927 as no. 577) grew in large clumps that could not be readily dispersed, and analytical results for cell carbon were not reliable. Hence, comparative results are recorded in table 3 for the Fleischmann strain (FY) and FF-16, a stock strain originally obtained from F. W. Tanner.

A comparison was also made between growth and metabolic activity in the glucose vitamin trace salt medium and on the same medium solidified by the addition of 2 per cent agar. Air was continuously circulated over 30 ml of the agar medium, which had a depth of 9 mm. Oxygen consumption was noted during the early stages of growth, but the R.Q. was considerably above unity (0 to 10 hr, 2.13; 10 to 21 hr, 2.76) for the first 24 to 30 hours, then fell to a value of 0.56 for the period 48 to 61 hours. Oxygen consumption and carbon dioxide

TABLE 2

Carbon balance during growth of S. cerevisiae

(Calculated on basis of 60 ml medium originally containing 252.7 mg C)

CARBON	TIME IN HOURS													
	16		23		27		40		52		64		72	
	mg	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%	*mg*	%
Cellular........	17.7	7.0	22.8	9.0	25.7	10.2	36.8	14.5	41.0	16.2	40.2	15.9	38.8	15.4
Supernatant....	141.8	56.0	99.5	39.4	73.9	29.2	31.2	12.4	14.4	5.7	11.9	4.7	10.9	4.3
Alcohol vaporized....	7.1	2.8	18.7	7.4	57.1	22.6	43.9	17.4	46.5	18.4	53.8	21.2	56.0	22.1
CO_2..........	35.9	14.2	78.4	31.1	72.7	28.8	93.5	37.0	113.2	44.8	141.1	55.9	151.2	59.8
Total recovered....	202.5	80.1	219.4	86.8	229.4	90.8	205.4	81.3	215.1	85.1	247.0	97.7	256.9	101.7

TABLE 3

Comparative carbon balance with two strains of S. cerevisiae and glucose or sucrose as substrate

	PER CENT ORIGINAL CARBON		
	FY		FF-16 Glucose
	Glucose	Sucrose	
Cells..................................	15.4	20.3	15.2
Supernatant.....	4.3	16.2	7.7
Alcohol vaporized..............................	22.1	27.4	25.2
CO_2...	59.8	37.0	55.9
Per cent recovery................................	101.6	100.9	104.0

production were negligible in the period 61 to 69 hours. The cells were then removed from the agar surface, washed by centrifugation, and cell counts and carbon determinations were made on representative samples. The agar was homogenized in 4 volumes of water in a Waring "blendor," and aliquots were used for the determination of volatile acids (none found), alcohol (0.62 mg), and reducing substances such as glucose. The carbon balance is reported in table 4.

The extent of carbon assimilation was somewhat higher on the agar medium than in the same medium without agar. The possibility exists that there may be a small amount of available carbon in the agar, but the carbon balance does

not support this suggestion. The cell crop per ml of medium was approximately three times greater on the agar medium than in the liquid medium, although single cell carbon was less, 0.5×10^{-8} mg as compared with 1.0 to 1.2×10^{-8} mg in the liquid medium.

Essentially the same type of results (see table 3) was obtained when glucose was replaced by sucrose as the energy carbon source. The maximum cell count was not quite as high, but the cells were observed to be larger and their carbon content tended to be approximately double that of the cells cultivated in the glucose medium. The amount of carbon assimilated was also slightly greater in the sucrose medium, approximately 20 per cent as compared with 15 to 16 per cent. A greater extent of assimilation from sucrose and maltose than from glucose had previously been observed with washed suspensions of yeast (Clifton, 1947).

With maltose as the substrate the results are of a preliminary nature. When a technical grade of maltose was used in the medium, the rate and extent of

TABLE 4

Carbon balance of S. cerevisiae cultivated on solidified synthetic medium

(Original glucose plus cell carbon = 137.45 mg)

CARBON	MG	PER CENT
Cellular........................	32.0	23.3
CO_2........................	69.7	50.7
Alcohol vaporized................	30.8	22.4
Glucose	7.0	5.1
Total........................	139.5	101.5

growth appeared to be somewhat higher and aerobic fermentation was not as pronounced as with glucose and sucrose. Less difference was noticed with a purified sample of maltose. These studies are being continued to determine whether a substance (or substances) is present in crude maltose that may act as a growth stimulant.

In experiments (Swanson, 1947) conducted under comparable conditions, the extent of assimilation during growth was determined. The percentages of carbon assimilated from various sugars, corrected for carbon in ethyl alcohol lost by vaporization, were as follows: crude maltose, 29 per cent; sucrose, 28 per cent; glucose, 20 per cent; and cp maltose, 16.5 per cent; in enriched synthetic medium and from glucose in the same medium solidified with agar, 30 per cent. In a number of experiments approximately 37 per cent of the carbohydrate carbon was evolved as carbon dioxide from crude maltose or cp sucrose as compared with 51 to 60 per cent from cp glucose or cp maltose. Ethanol lost from the medium by aeration ranged from 22 to 27 per cent of the carbohydrates dissimilated, with the exception of crude maltose, with which the loss was only 12 per cent. Microscopic examination of the cells from the various media suggested that crude maltose may contain a factor that stimulates budding; the cells from the sucrose

medium tended to be larger than those in glucose or maltose.   A greater tendency for the cells to adhere to each other was noted in the maltose medium.

Since carbon dioxide production is much greater than oxygen consumption in young cultures of *S. cerevisiae* in the synthetic medium, it was necessary to determine if the age of the culture or the nature of the medium influenced respiration of washed cells.   Accordingly, 14-, 24-, and 48-hour cultures in the synthetic medium were harvested by centrifugation, and the respiratory activity of these cells was tested by the Warburg technique.   Cells from the 14-hour cultures consumed relatively little oxygen but did produce considerable amounts of carbon dioxide, both aerobically and anaerobically.   Cells from older cultures consumed oxygen more readily and with little evidence of aerobic fermentation as evidenced by an R.Q. near unity.   Suspending the cells in the synthetic medium, in Kreb's saline, or in M/15 phosphate buffer, had no apparent influence on the rate of respiration.   Hence it appears that the nature of the respiratory activity of washed cells varies with the age of the parent cultures or, more to the point, with the presence or absence of glucose in the culture medium at the time the cells were harvested.   This behavior is being studied in more detail.

It appears that the normal mode of utilization of glucose in young aerobic cultures in the synthetic medium may be primarily by way of alcoholic fermentation, with subsequent oxidation of the alcohol as glucose disappears from the medium.

In a growth experiment of six hours' duration with glucose as the carbon source, respiration was entirely fermentative.   Carbon dioxide and ethyl alcohol accounted for 63.5 per cent of the glucose disappearing from the medium in a molar ratio of 3.16 to 4.13.   This suggests that a considerable portion of the carbon dioxide produced during fermentation is assimilated by the cells.   Only 13.3 per cent of the glucose utilized was accounted for as cellular material, indicating that with proliferating cells during the first stages of glucose utilization, 23.2 per cent of the sugar was transformed into products other than carbon dioxide, ethyl alcohol, and cellular material.   Glycerol and nonvolatile acids may, in part at least, account for much of this decrease in reducing sugar content of the medium.   Therefore additional studies on the oxidative assimilation of glucose and of alcohol by the Fleischmann strain of yeast were made.

Yeast cells cultivated without aeration in the synthetic medium for 24 hours were washed and suspended in Krebs's saline phosphate buffer of pH 5.0, and carbon dioxide production in M/200 glucose was determined by the Warburg technique.   Under anaerobic conditions, carbon dioxide production of 75 per cent of the theoretical value for complete fermentation was observed together with an increase of cellular carbon of from 0.13 to 0.14 mg as compared with a postulated assimilation of 0.18 mg based on the equation

$$2C_6H_{12}O_6 \rightarrow 3(CH_2O) + 3C_2H_5OH + 3CO_2$$
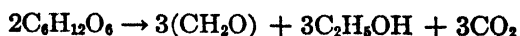
Under aerobic conditions the increase in cellular carbon was 0.36 to 0.38 mg as compared with 0.48 mg postulated by the equation for oxidative assimilation of glucose:

$$C_6H_{12}O_6 + 2O_2 \rightarrow 4(CH_2O) + 2CO_2 + 2H_2O$$

Similar quantitative relationships were observed with cells from Sabouraud's agar.

The oxidative assimilation of alcohol involves an oxygen consumption of approximately 58 per cent of that required for complete combustion and a carbon dioxide production of 47 per cent with an R.Q. of 0.54 (theoretical 0.66). This would suggest

$$C_2H_5OH + 2O_2 \rightarrow (CH_2O) + CO_2 + 2H_2O$$

as an approximate equation for the oxidative assimilation of alcohol by yeast. Experimentally an assimilation by washed cells of as much as 0.58 mg of carbon (theory 0.6 mg) has been observed from 1.2 mg of ethyl alcohol carbon. In macro growth experiments with ethyl alcohol as the sole substrate the rate of growth was so slow that the results are of little value. However, in the glucose medium a rapid decrease in alcohol content was observed, together with oxygen consumption and increases in cell count and cellular carbon, after glucose disappeared from the medium. This indicates that alcohol can be oxidatively assimilated by proliferating cultures of yeast.

DISCUSSION

The first stage in the utilization of glucose, sucrose, or maltose in an aerated vitamin-salt medium by proliferating cultures of the strains of *S. cerevisiae* here employed was found to be one of fermentative assimilation. Little or no oxygen was utilized in this initial stage, and some indication that oxygen exerted an inhibitory action was observed. Furthermore, washed cells from young cultures did not consume oxygen with any of the above-mentioned sugars as the substrate, but did ferment them aerobically, whereas cells from older cultures readily utilized oxygen. Fermentative assimilation dominated over aerobic oxidation as long as there was an appreciable quantity of reducing sugar in the synthetic culture medium. The rate of cellular proliferation and synthesis of cellular carbon was greatest during the period of fermentative assimilation.

Although Lundin (1923) reported assimilation by yeast cells without proliferation when ethanol was used as the substrate, whereas Stier (1939) recorded that ethanol is apparently not used for carbohydrate synthesis, our results indicate on the basis of the comparatively large quantities of ethanol (as an intermediate from the utilization of glucose) disappearing from the medium that both respiration and assimilation occurred together with proliferation. In other studies to be reported later it has been observed that ethanol as the carbon and energy source will support aerobic growth of the Fleischmann strain of *S. cerevisiae*, and that although the rate of growth on ethanol is much lower than on the various sugars tested, it may be increased to some extent by serial passage in the ethanol medium.

When relatively large inocula are employed, fermentative assimilation may be suppressed to some extent by oxidative assimilation. This may be due, at least in part, to a closer association of the cells with each other. This concept is supported by the observation that growth is more rapid in unstirred cultures or

on a solid medium.   Carbon dioxide, however, did not appear to be the limiting factor.

   Pickett and Clifton (1943) reported an oxidative assimilation of approximately one-third of the glucose carbon by washed cells.   In this study the over-all assimilation, corrected for alcohol lost by evaporation, was only about one-fifth of the glucose carbon disappearing from the medium.   These figures are further apart than would appear at first glance.   With washed cells in experiments of short duration, approximately one-third of the glucose carbon remains in the medium in end products of glucose dissimilation that are not readily oxidized, whereas only 4 to 8 per cent of the glucose carbon is present in end products at the end of several days' incubation of the cultures.   Therefore, although synthesizing less cellular carbon, the proliferating organism makes use of more of the end products of glucose dissimilation than does the nonproliferating cell.

   Some evidence was obtained that suggests that up to 40 per cent of the carbon assimilated during the fermentative assimilation phase of growth might be from carbon dioxide.   This is based on the discrepancy between molar quantities of ethanol and carbon dioxide produced in the young cultures, equimolar quantities being produced during fermentative assimilation by washed cells.

   As pointed out by Pickett and Clifton (1943), no simple equation, as derived from manometric data, can represent the basic assimilatory process. · Studies such as this demonstrate that respiration and assimilation during different periods of growth may involve the utilization of different types of intermediate products of carbohydrate dissimilation.   This becomes most apparent with an organism such as yeast in a synthetic medium in which marked discrepancies are noted in both R.Q. (infinity to as low as 0.25) and extent of assimilation on comparing results obtained during growth with those observed with washed suspensions. The R.Q. and the extent of assimilation in washed suspensions and in proliferating cultures are more nearly the same with *Escherichia coli* as the test organism.

   The foregoing observations suggest that the nature of the medium may also have considerable effect on the quantitative relationships between assimilation and dissimilation.   The growth of yeast in the aerated synthetic medium was not as rapid as in unstirred cultures.   This was shown not to be due to the removal of carbon dioxide from the medium by aeration.   Possibly the nitrogen source, ammonium salts, was not entirely suitable for growth in aerated cultures. For example, Brandt (1945) reported that aerobic fermentation of glucose was considerably increased in the presence of ammonium chloride and was weak in its absence.   However, no appreciable difference was noted in studies to be reported elsewhere when casein hydrolyzate was substituted for ammonium salts.

   The enhanced growth and aerobic respiration observed when technical maltose, but not cp maltose, was employed as the substrate further support the conclusion that the synthetic medium employed in this study might be deficient in one or more factors essential for the rapid growth of yeast in aerated cultures.   Williams *et al.* (1936) concluded that the greater glycogen storage by yeast-utilizing maltose was due, at least in part, to the pantothenic acid content of maltose.   However, doubling or quadrupling the pantothenate concentration had little or no

influence on the growth of the Fleischmann strain in the aerated cultures.   Likewise, no known growth factor (to be reported elsewhere) enhances growth to the extent observed following the addition of small amounts of technical maltose to the glucose-containing medium.   Hence it appears that there may be one or more growth-stimulating agents present in technical maltose and possibly in the other more complex media frequently employed for the cultivation of yeast. The agent in technical maltose does not appear to enhance assimilation by washed cells, which we may tentatively assume have synthesized the factor, but it does enhance assimilation by proliferating cells.   This may be the result of its growth-stimulating properties.

SUMMARY

Actively proliferating cultures of certain strains of *Saccharomyces cerevisiae* when cultivated aerobically carry on the most active initial stages of their metabolism, from the point of view of growth rate and synthesis of cellular carbon, by the process of fermentative assimilation.   This stage predominates until hexose or other polyose molecules disappear from the medium.   Oxidative assimilation then occurs from intermediate products of saccharide dissimilation, including ethanol, until practically all of these have been utilized with the exception of the nonvolatile organic acid(s).

A comparatively greater percentage of synthesis occurs from the disaccharides sucrose and crude maltose (but not cp maltose) than from glucose.   Some evidence was obtained that this may be induced by the presence of an unknown growth factor in the sugars and particularly in the crude maltose employed.

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THE INFLUENCE OF CERTAIN SUBSTANCES ON THE ACTIVITY OF STREPTOMYCIN

III. DIFFERENTIAL EFFECTS OF VARIOUS ELECTROLYTES ON THE ACTION OF STREPTOMYCIN

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The activities of both streptothricin and streptomycin are affected by substances and physical conditions that apparently do not affect these compounds per se. Foster and Woodruff (1943) noted that the presence of phosphates, some other salts, and sugars in test media decreased the activity of streptothricin. Similar observations with regard to streptomycin were presented by Waksman and Schatz (1945). Foster and Woodruff (1943) concluded that phosphates and sugars caused the observed interference through effects on pH, since it was also found that raising the pH of the medium increased the streptothricin activity and vice versa. They concluded that only the undissociated free base of this antibiotic was active, hence the increased activity at a higher pH. Since streptomycin responded similarly to pH changes (Waksman and Schatz, 1945), presumably one might apply the same deductions in this case.

Abraham and Duthie (1946), on the other hand, suggested the possibility of bacterial cell surface changes accompanying change in pH and posed the question of whether there might not be competition at the surface between hydrogen ions and the cations of dissociated streptomycin, hence the lower antibiotic activity at lower pH's.

In reporting the interfering action of sugars on streptomycin activity, Waksman and Schatz (1945) observed that such effects were probably caused by pH changes in the medium, but they also discussed the possibility of the reducing role played by sugars. Later, Geiger, Green, and Waksman (1946) concluded that the effects of sugars were after all caused by pH changes, since glucose was found to have no effect on streptomycin in the presence of a glycine buffer. This conclusion was questioned by Donovick and Rake (1946) because increased glucose oncentrations, under the conditions of the test, did not lead to a lower pH but did decrease the activity of the antibiotic. In addition, the degree of glucose effect varied with the concentration of tryptone in the medium.

The explanation for the effects of salts other than phosphates on the action of streptomycin remains equally obscure. Berkman, Henry, and Housewright (1947) stated that the capacity of various salts to cause this interference was a function of the salt concentration. They reported little interference by salts at concentrations below 0.5 per cent. This antagonism was not due to stimulation of the growth of the test organism by the change of salt concentration.

They included several species of microorganisms and six different salts in their studies, and concluded that the results with all salts were alike, differences being only quantitative.

These authors proposed two possible explanations for the antagonism of salts to streptomycin action: interaction between salt and streptomycin, or the result of an interaction of salt and bacteria. They were unable to demonstrate any interaction between streptomycin and the salts studied. As to the possible nature of the interaction between salts and bacteria, no proposals were extended.

Another possible explanation for the action of salts, as well as one for a mode of action of streptomycin, was brought forward by Cohen (1946, 1947), who found that desoxyribonucleic acid (DNA) was precipitated by streptomycin and that this precipitate was caused to dissociate by the addition of salts. Since bacteria contain DNA, it was thought that perhaps streptomycin interfered with some normal cell functions through such precipitation. This action of streptomycin on DNA and its reversal by salts was confirmed by Berkman *et al.* (1947).

A different approach to the question of the interference by certain salts on the antibiotic activity of streptomycin was taken by Green (1947). This author reported that salts of pyruvic, fumaric. succinic, formic, malic, and maleic acids all interfered with the action of this antibiotic, whereas lactate, acetate, propionate, glycerophosphate, as well as glycerol and lactose, had no antagonizing effect. This work was in line with the thought that the mode of action of streptomycin was in some way tied to the oxidative mechanisms of susceptible microorganisms. (Since this aspect of possible modes of action of streptomycin is outside the scope of the present paper, the literature dealing with it is not reviewed here. See Geiger, Green, and Waksman, 1946; Bondi, Dietz, and Spaulding, 1946; Donovick and Rake, 1946; etc.) Organic salts such as Green found to interfere with streptomycin action were thought to counteract the action of streptomycin by supplying some necessary intermediates in carbohydrate metabolism that are blocked by streptomycin.

The mode, or modes, of action of streptomycin and other antibiotic substances has attracted the attention of many laboratories, and numerous approaches to the problem have been chosen. The manner in which electrolytes affect the action of streptomycin was felt in this laboratory to offer an interesting point of attack on the problem. Relatively little is known about the effects of electrolytes on bacteria. The literature on this subject is tremendous, and no attempt can be made here to cover it. Porter (1946) provides a selected bibliography on this complex subject. Falk (1923) thoroughly reviewed the literature prior to 1923. A comment made by this author almost 25 years ago unfortunately still holds true today: "The basis of knowledge upon which rests the use of synthetic media is still essentially empirical. The physiological significance of this or that ion is not known and cation—or anions, for that matter—are thrown in or taken out of media according to whether growth is good, bad or indifferent."

Falk's paper covered both the physiologic and physicochemical aspects of the problem. An interesting study on the latter, insofar as adsorption of various cations by bacteria is concerned, was published by McCalla in 1940. Some of

his methods might well be applied to the investigation of the manner in which electrolytes compete with streptomycin. Do such electrolytes prevent streptomycin from reaching the vital site of reaction, or do they interfere with the final reactions in which streptomycin enters in causing inhibition? To attempt to answer some of these questions is the over-all purpose of the present work.

It appeared to the authors that it was necessary first to gather more exact data on the effects of electrolytes on the growth-inhibiting action of streptomycin on bacteria, and then, later, using this information as a baseline, to attempt to correlate it with data gathered from experiments specifically designed to test various theories of the mode of action of this antibiotic. *Klebsiella pneumoniae* (ATCC 9997) was chosen as the test organism because considerable information had previously been obtained on its response to streptomycin, and on the assumption that in general the mode of action of streptomycin was the same regardless of the test organism. Whether or not such an assumption is valid remains to be seen.

PROCEDURE

The basic medium used throughout this portion of the work consisted of 0.75 per cent tryptone in water, which upon autoclaving is found to have a pH very close to 7. Electrolytes that could withstand autoclaving at 15 pounds pressure for 15 minutes were incorporated into the medium before sterilization. Otherwise, the electrolyte solutions in 1-molar or 2-molar solution were sterilized by filtration through U.F. fritted Corning pyrex glass filters, and appropriate amounts were added to sterile tryptone broth to give the desired final concentrations of salt.

The trihydrochloride of highly purified streptomycin A[1] (Fried and Titus, 1947) was used throughout the work described. To establish the minimal inhibiting concentration of streptomycin in the various media employed, the procedure was that described previously (Donovick and Rake, 1946). All M.I.C. figures reported are based on no less than eight assays, giving a standard error of approximately ±6 per cent.

Differentiation between the Effects of Phosphate Ion and Hydrogen Ion on the Activity of Streptomycin

Buffer solutions consisting of NaH_2PO_4-Na_2HPO_4 were prepared so as to give final concentrations of phosphate in tryptone broth ranging from 0.004 M to 0.067 M and at each concentration from pH ca. 6.0 to pH ca. 7.5. In addition, as controls, tryptone broth was adjusted with NaOH or HCl over a range of pH 6 to 9. The M.I.C. of streptomycin A trihydrochloride was then determined in each of these media. The results, shown in figure 1, indicate that the effect of phosphate ion can be readily distinguished from that of hydrogen ion. At each concentration of phosphate, an increase in pH from 6 to 7.5 caused a 3- to 4-fold decrease in M.I.C. At any fixed pH an increase in phosphate concentration caused a relatively consistent increase in M.I.C.

The Behavior of Streptomycin in Vitro in the Presence of Various
Electrolytes   at   Constant   pH

Since the effects of phosphate ions could be distinguished from those of hydrogen ions on the action of streptomycin A, it appeared reasonable to expect the
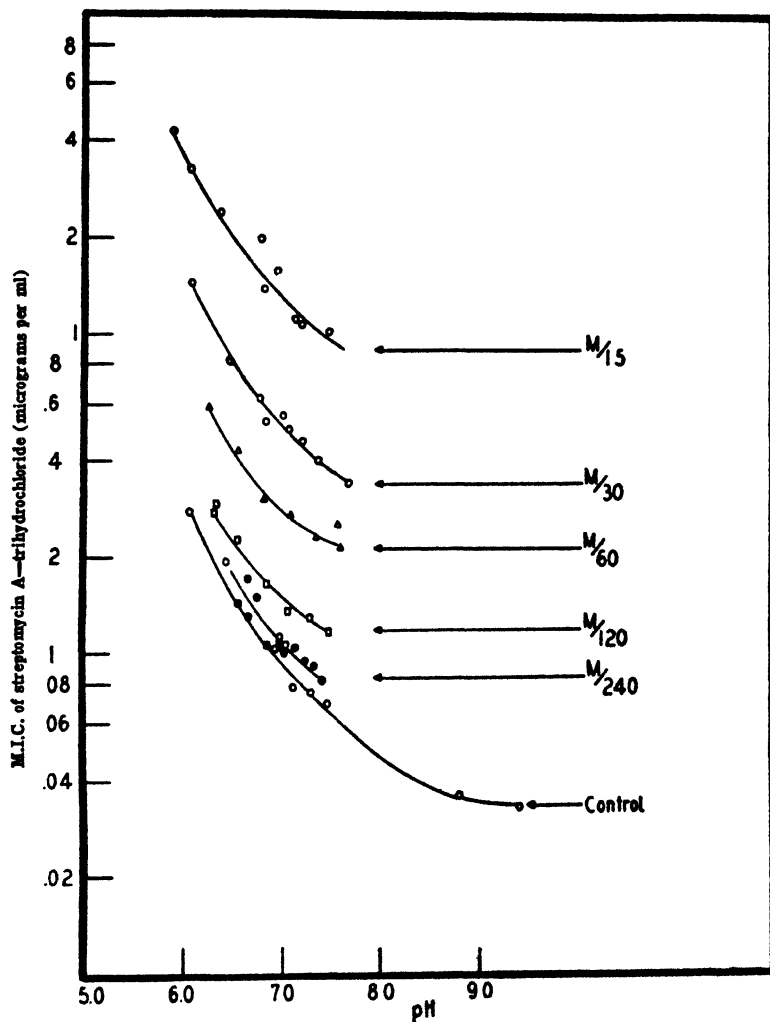

Figure 1. Effect of phosphate on streptomycin activity at various pH's.

effects of other ions to be distinguishable as well.   Hence studies including 17 different salts were undertaken, each at concentrations ranging from 0.004 M through 0.067 M.   For studying anions, sodium was generally the cation of the salt used.   For studying cations, on the other hand, chloride was generally the

anion employed.   Since the pH of the test medium exerts an effect on strep-
tomycin action separate from that of other ions, it was desirable in this study to
work within as narrow a pH range as possible.   To this extent the pH of the
0.067 M sodium pyruvate was too high, and those of 0.067 M sodium lactate,
barium chloride, magnesium chloride, and calcium chloride in 0.75 per cent
tryptone broth were too low to be entirely satisfactory.   Nevertheless, the results
of these studies, presented in figure 2, indicate that sodium ion, per se, has little
effect on the action of streptomycin, as exemplified by the results with sodium
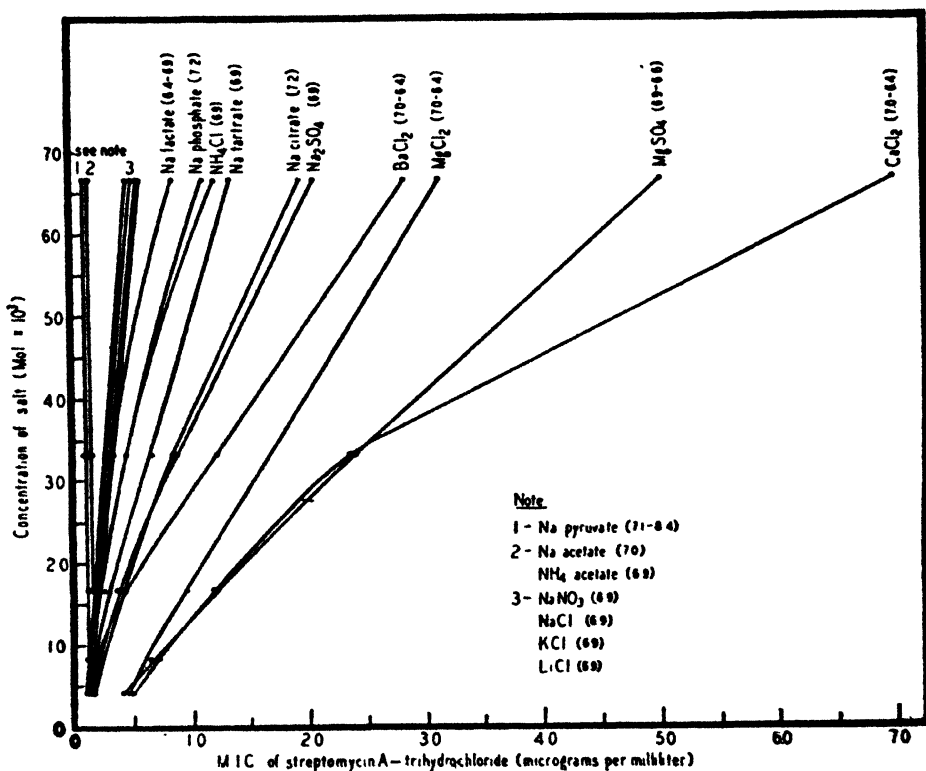
Figure 2. Effect of various salts on streptomycin A action *in vitro*.

acetate and pyruvate, nor do lithium and potassium ions, since their chlorides
are equal in activity to that of sodium chloride.   Ammonium presents a puzzling
problem because as an acetate it causes no interference, whereas as a chloride its
action is greater than that of sodium chloride.   Magnesium and calcium have
very marked effects, and magnesium sulfate represents an interesting example of
the additive action of two active ions (viz., Mg^{++} and SO^{--}) causing greater
interference than either sodium sulfate or magnesium chloride.

Barium chloride and calcium chloride presented special problems in concen-
trations of 0.067 and 0.033 M since they caused precipitates in the medium.   The

presence of such precipitates did not interfere seriously with the tests since they settled out and could be distinguished from the bacterial growth. Removal of the barium precipitates, by filtration, prior to use caused very little change in the results. However, when the precipitates caused by calcium chloride were filtered off, the pH of the broth dropped from pH 6.4 (unfiltered) to 6.2 and the M.I.C. rose accordingly. Hence the results shown in figure 2 are those obtained with the precipitate present.

Among the anions, acetate and pyruvate show little or no effect, whereas, in increasing order, the following interfere with streptomycin action: nitrate, chloride, lactate, phosphate, tartrate, citrate, and sulfate.

The explanation for the order of activities of these ions is not at once evident. At first glance one is tempted to associate ionic strength with the ability to cause interference. There are sufficient contradictions, however, to cause one to abandon this principle. Comparison of $CaCl_2$ and $MgCl_2$, the ionic strengths of which would be very similar in equimolar concentrations, exemplifies such a contradiction. It is the hope that future work will cast more light on the observed phenomena.

Effect of Salts on the Precipitation of Desoxyribonucleic Acid (DNA) with Streptomycin A

Cohen (1946, 1947) has described the precipitation of desoxyribonucleic acid by streptomycin. Further he has shown that streptomycin also precipitates bacteriophage T_r—F, which is rich in DNA. Other pertinent points brought out in these publications are that desoxyribonuclease (DNase) depolymerizes the DNA present in this bacteriophage with a consequent decrease in viscosity but without inactivation of the bacterial virus. Streptomycin, on the other hand, precipitates the desoxyribonucleic acid and also inactivates the bacteriophage. Hence the possibility was presented that streptomycin acts through such precipitation. The description of the dissociation of streptomycin-DNA precipitates by salts (Cohen, 1946, 1947; Berkman et al., 1947) led the present authors to attempt to correlate the ability of various ions to interfere with the growth-inhibitory action of streptomycin with the power of these ions to prevent the precipitation of DNA with streptomycin. For this study the authors are indebted to Dr. Samuel Graff, of the College of Physicians and Surgeons, Columbia University, for a highly purified, relatively salt-free preparation of DNA.

Precipitation of desoxyribonucleic acid by streptomycin. A simple method was employed for measuring the precipitability of DNA with streptomycin. The minimal concentration of streptomycin A trihydrochloride required to give a visible precipitate of DNA was determined by carefully adding to 1.5 ml of 0.1 per cent aqueous solution of DNA 1.5 ml of various concentrations of streptomycin in such a fashion as to give a ring. Although precipitates formed at once when adequate amounts of streptomycin were present, final readings were not made until the tests had stood at 4 C overnight in order to ensure full development of the ring. Similar tests were carried out to determine the minimal con-

centration of DNA required to give visible precipitates at a fixed streptomycin concentration.   In this case a 0.5 per cent streptomycin solution was used.   The findings were as follows: DNA added in a concentration of 0.1 per cent showed a visible precipitate with streptomycin A trihydrochloride added in a concentration as low as 0.032 per cent.   The minimal concentration of DNA showing a precipitate when added to 0.5 per cent streptomycin A trihydrochloride was 0.0032 per cent.   For further work described below, both of these reagents were used in large excess as measured by this test.

Effect of phosphate and hydrogen ions on the precipitation of DNA with streptomycin.   A series of Na_2HPO_4-NaH_2PO_4 buffer solutions were prepared ranging from 0.268 M to 0.016 M (i.e., four times as concentrated as desired in the final concentration in the test in which a 4-fold dilution of the buffer solutions was involved), and at each concentration the pH ranged from 5.2 to 7.4.   To 0.125 ml of buffer solution 0.25 ml of 0.25 per cent aqueous DNA solution were added and thoroughly mixed.   To this 0.125 ml of 0.5 per cent aqueous solution of streptomycin A trihydrochloride were then carefully added to form a ring.   All tests were permitted to stand overnight at 4 C to permit full formation of pre-

TABLE 1

Minimal phosphate concentration preventing DNA-streptomycin A precipitates at various pH's

pH	MINIMAL PHOSPHATE CONCENTRATION
	molarity
5.2	0.062
5.9	0.062
6.4	0.047
6.8	0.031
7.4	0.023

cipitate in border-line cases.   Thus with streptomycin and DNA concentrations held constant, the minimal phosphate concentrations preventing precipitation at various pH's were measured.   These results are given in table 1.

These findings at once reveal an interesting contrast between the manner in which phosphate and hydrogen ions affect the DNA-streptomycin precipitation system as compared to the streptomycin-growth-inhibiting system.   Since in the former system the concentrations of DNA and streptomycin were held constant, it was necessary, for purposes of comparison, to pick from figure 1, shown earlier, similar data.   In the bacterial system the microorganisms themselves were the only source of DNA, and as the inoculum was kept constant in all cases, this criterion had been met.   In order to obtain data for the effect of phosphate and hydrogen ions on the bacterial system at constant streptomycin concentrations, however, extrapolation from the curves in figure 1 was necessary in some cases.   For this comparison the point at which each pH-phosphate curve crosses the M.I.C. line of 0.4 micrograms per ml in figure 1 was determined, extrapolating where necessary.   Although a certain amount of error is involved in this procedure, considering the nature of the curves involved, it was felt that the relative

values thus derived were valid.　The data thus obtained and those shown in the foregoing table are plotted in figure 3.

It is evident that increasing the pH, which increases the antibiotic activity of streptomycin, causes an increase in the concentration of phosphate required to prevent the antibacterial action of streptomycin.　On the other hand, a similar increase in pH in the DNA-streptomycin precipitation system causes a decrease in the amount of phosphate required to prevent precipitation.　This point of difference having been established, it was of interest to compare how various other ions behave in the two systems described.
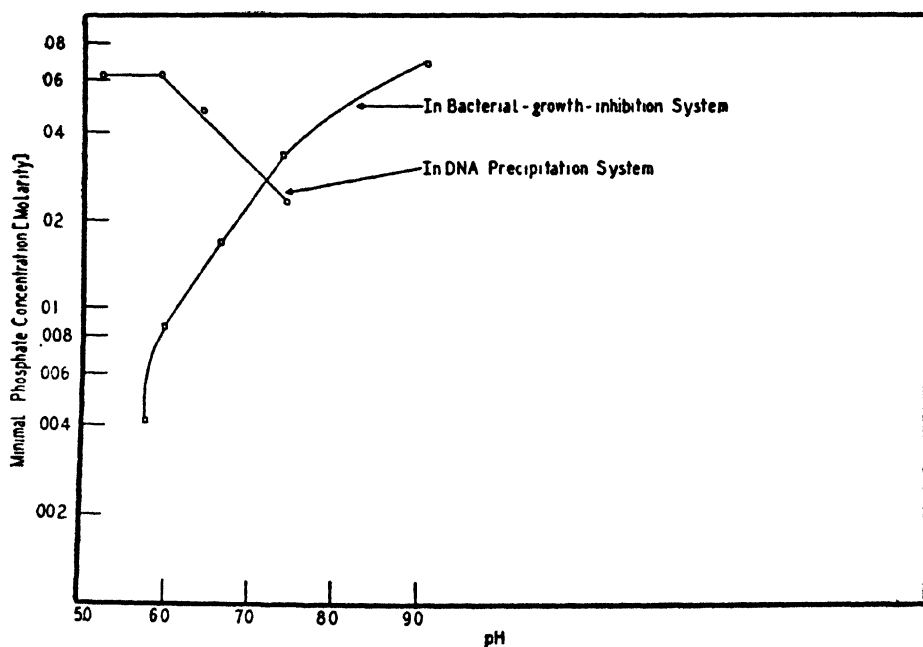

Figure 3.　Interrelationship between effects of phosphate and hydrogen ions at fixed streptomycin concentrations.

Effects of various other ions on the DNA-streptomycin precipitation system.　A series of aqueous salt solutions were prepared ranging in concentrations from 0.268 M to 0.01 M.　The minimal salt concentration preventing the formation of a DNA-streptomycin precipitate was determined in the manner described above. At the same time, from figure 2, the approximate point at which each salt-concentration-streptomycin-M.I.C. curve crossed the M.I.C. line of 1.5 micrograms per ml was determined by extrapolation.　Since the curves for all salts less active than sodium tartrate in preventing the growth-inhibiting action of streptomycin approach straight lines, it was felt that the error involved here was not prohibitive for comparative purposes.　This comparison is shown in table 2.

In the cases of some of the uni-univalent salts such as sodium chloride, sodium

nitrate, and potassium chloride, the relative action of the salts is similar in both systems.   However, there are also cases of marked differences of behavior.   In the DNA-streptomycin precipitation system, for example, no distinction is evident between the relative actions of sodium sulfate and calcium chloride, whereas in the prevention of the inhibition of bacterial growth the latter salt is twice as active as the former.

Of further interest are the differences in behavior of some of the salts of organic acids in the two systems, especially sodium citrate.   One wonders whether the relatively low activity of such salts in their ability to interfere with the growth-

TABLE 2

Comparative action of salts in preventing growth inhibition of K. pneumoniae as against preventing DNA-streptomycin-A precipitation

SALT TESTED	MINIMAL SALT CONCENTRATION			
	Prevention of growth inhibition		Prevention of precipitation	
	Absolute	Relative*	Absolute	Relative*
	molarity		*molarity*	
Calcium chloride............	0.023	9.6	0.031	4.0
Magnesium chloride.................	0.029	7.6	0.031	4.0
Barium chloride....................	0.040	5.5	0.063	2.0
Sodium sulfate....................	0.052	4.2	0.031	4.0
Sodium citrate....	0.055	4.0	0.0059	21
Sodium tartrate.	0.076	2.9	0.031	4.0
Ammonium chloride.......	0.098	2.2	0.125	1.0
Sodium phosphate	0.10†	2.2	0.031‡	4.0
Sodium lactate.....	0.13	1.7	—	—
Sodium nitrate	0.22	1.0	0.125	1.0
Sodium chloride	0.22	1.0	0.125	1.0
Potassium chloride.	0.22	1.0	0.125	1.0
Lithium chloride	0.22	1.0	0.125	1.0
Sodium acetate.......	0		0.125	1.0
Ammonium acetate.. .... } No effect	0		0.125	1.0
Sodium pyruvate..	0		0.031	4.0

* As compared to sodium chloride taken as 1.0.
† Tested at pH 7.2.
‡ Tested at pH 6.8.

inhibiting action of streptomycin may be due to the utilization of the anion by the bacteria.   Preliminary tests on this point were conducted in a synthetic medium in which sodium lactate is ordinarily the sole source of carbon (Cohen, 1947). This medium contains 1 g of NH_4Cl, 0.7 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.1 g of Na_2SO_4, 0.01 g of $MgSO_4$, and 10 g of sodium lactate per liter of distilled water. The strain of *K. pneumoniae* used throughout these studies grows readily in this medium at least through 10 transfers.

To test the utilization of organic acid salts other than lactate, media were prepared in which 0.09 and 0.18 M sodium acetate, ammonium acetate, sodium pyruvate, sodium tartrate, and sodium citrate, respectively, were substituted

for sodium lactate. Ten ml of each of these media were inoculated with 0.1 ml of an overnight culture grown in the synthetic lactate medium. Each of these cultures was then incubated for 1 week at 37 C, observations for visible turbidity being made daily. Lactate controls were included with each day's tests. Usually no visible growth occurred in any of the media other than the controls, although sparse growth occurred once in the ammonium acetate, once in the sodium tartrate, and once in the sodium citrate media. There were still viable organisms present in all media at the end of 1 week, however, as shown by streak plates.

It is recognized, of course, that the inability of these salts to substitute for lactate in the synthetic medium used is no final measure of whether they are utilized by *K. pneumoniae* under other circumstances. This is shown in at least the case of sodium pyruvate, which when added in a concentration of 0.2 M to 0.75 per cent tryptone broth is fermented by *K. pneumoniae* with a consequent pH drop in 24 hours. Such acid formation with the other salts did not occur, and, hence, other types of tests for their utilization are necessary. These have not yet been performed.

On the basis of the behavior of streptomycin in the presence of phosphates and a number of other ions, it must be concluded that no good correlation is found between the precipitation of DNA and the prevention of bacterial growth. To test this even further still another approach was attempted.

Action of DNase on DNA-streptomycin precipitates. Aqueous solutions of DNA have a high viscosity, which is readily reduced by desoxyribonuclease (DNase) (McCarty, 1946). Cohen (1946, 1947) reported that Na DN (desoxyribonucleate) thus depolymerized by DNase "did not reduce the precipitability [with streptomycin] of the resulting Na DN after 1 hour of depolymerization." However, the present authors found that when DNA-streptomycin precipitates are treated with adequate amounts of DNase, dissolution of the precipitates occurred. (We wish to express our appreciation to Dr. M. Kunitz of the Rockefeller Institute, Princeton, New Jersey, for the crystalline DNase used in the following experiments.)

DNase solutions were prepared in 0.05 M $MgCl_2$ containing 0.25 per cent gelatin, under which conditions the enzyme is relatively stable when stored at 4 C (McCarty, 1946). With 0.025 M $MgCl_2$ containing 0.25 per cent gelatin as the diluent, the enzyme solution was diluted serially by 2-fold steps. To 0.125 ml of the various dilutions of enzyme were added, in turn, 0.125 ml of 0.5 per cent streptomycin A trihydrochloride and 0.25 ml of 0.025 per cent DNA. Readings were made for the presence or absence of precipitate immediately, and after incubating at 37 C over the time intervals shown in table 3. The experiment shown is one example of a number of repeat tests that were conducted.

It can be seen, thus, that DNA precipitated by streptomycin can still be depolymerized by DNase, with consequent disappearance of the precipitate. If the precipitation of DNA by streptomycin is a vital part of the manner in which this antibiotic inhibits bacterial growth, then one might expect DNase to interfere with the growth-inhibiting action of streptomycin. That DNase is capable

of entering at least into the dead bacterial cell and depolymerizing DNA has been demonstrated with *E. coli*, *Bacillus anthracis*, and *Corynebacterium diphtheriae* (Tulasne and Vendrely, 1947), as well as with the same strain of *K. pneumoniae* as has been used throughout these studies (Rake and Hamre, to be published). Hence, tests were set up to determine the M.I.C. of streptomycin in the presence of an excess of DNase.[2] For this purpose 0.75 per cent tryptone broth containing 0.003 M MgCl$_2$ was used to ensure the presence of adequate amounts of Mg^{++} to activate the enzyme. The DNase concentration of the broth was then brought to 0.5 µg per ml, the broth was inoculated with *K. pneumoniae*, and streptomycin titrations were run as usual. No significant differences were found

TABLE 3

The action of DNase on DNA-streptomycin A precipitates at 37 C

TUBE	DNase CONC.	STM. CONC.	DNA CONC.	RESULTS IN X HOURS									
				0	0.25	0.5	0.75	1	1.5	2	2.5	4.5	24
	µg/ml	%	%										
a	0	0.125	0.0125	+	+	+	+	+	+	+	+	+	+
b	28.7	0.125	– –	–	–	–	–	–	–	–	–	–	
1	14.3	0.125	0.0125	+	–	–	–	–	–	–	–	–	–
2	7.2	"	"	+	±	–	–	–	–	–	–	–	–
3	3.6	"	"	+	+	–	–	–	–	–	–	–	–
4	1.8	"	"	+	+	±	±	–	–	–	–	–	–
5	0.90	"	"	+	+	±	±	–	–	–	–	–	–
6	0.45	"	"	+	+	+	±	±	–	–	–	–	–
7	0.22	"	"	+	+	+	±	±	±	±	±	f. tr.	–
8	0.11	"	"	+	+	+	+	±	±	±	±	f. tr.	–
9	0.056	"	"	+	+	+	+	±	±	±	±	±	–
10	0.028	"	"	+	+	+	+	+	±	±	±	±	–
11	0.014	"	"	+	+	+	+	+	+	+	+	+	±
12	0.0070	"	"	+	+	+	+	+	+	+	+	+	±

+ = precipitate approximately as heavy as control tube a.
± = precipitate present but lighter than controls.
f. tr. = very faint trace of precipitate.
– = no precipitate visible.

in the M.I.C.'s of streptomycin A between broths conaining DNase or lacking it, the former being 0.27 µg per ml and the latter 0.26 µg per ml. Not even at 10 times this concentration of DNase (i.e., at 5 µg per ml) were there significant differences.

Thus, as in the case of the behavior of streptomycin in the presence of salts, its behavior in the presence of DNase does not confirm the theory that this antibiotic

[2] First the effect of DNase itself on the growth of *K. pneumoniae* in this broth was tested. Tulasne and Vendrely (1947) had described the DNA present in several species of bacteria as being nuclei. It was anticipated, therefore, that perhaps the growth would be inhibited. Concentrations of crystalline DNase as high as 5 µg per ml, however, had no apparent effect on the rate of multiplication of our test organism.

acts by precipitating the DNA in the living cell. Other observations also make this theory improbable. It must be pointed out that T_2—F bacteriophage is not a good example from which to draw conclusions. DNA is not always present as a constituent of this bacteriophage, its presence or absence depending entirely upon cultural conditions; also it can be removed by DNase without interfering with phage activity; and yet streptomycin does inactivate the bacteriophage. Furthermore, as has been pointed out by Hamre and Rake (1947), streptomycin has no activity on one group of agents—the *Chlamydozoaceae* (lymphogranuloma-psittacosis group)—members of which are known to be particularly rich in DNA.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. Geoffrey Rake, Division of Microbiology, The Squibb Institute, for his many kind criticisms and suggestions in connection with this work.

SUMMARY

Quantitative studies of the effects of various ions on the action of streptomycin on bacterial growth as well as on its ability to precipitate desoxyribonucleic acid have been conducted.

Sodium, lithium, and potassium ions have little effect on the ability of streptomycin to inhibit bacterial growth.

Ammonium ion presents a puzzling problem since as an acetate it appears to have no effect on the growth-inhibiting action of streptomycin, whereas as a chloride its action is greater than can be accounted for by the action of the chloride ion alone.

Of the six cations studied, magnesium and calcium caused the greatest interference with streptomycin activity.

Among the anions studied, acetate and pyruvate caused little if any interference, whereas in increasing order the following were active in interfering with the prevention of growth by streptomycin: nitrate, chloride, lactate, phosphate, tartrate, citrate, and sulfate.

If both the cation and anion of a given salt show this interference, their effects may be additive, as is exemplified by magnesium sulfate.

Various salts also interfere with the precipitation of desoxyribonucleic acid by streptomycin, but the order of activity of the salts is different from that in the interference with the growth-inhibiting action of streptomycin. This and also studies with the enzyme desoxyribonuclease lead to the conclusion that the ability of streptomycin to precipitate desoxyribonucleic acid has little to do with the antibacterial action of this antibiotic.

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THE INHIBITING EFFECT OF NORMAL SERUM AND ITS GAMMA GLOBULIN FRACTION UPON THE VARIATION OF STAPHYLOCOCCUS AUREUS

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The modifying or selective effect of environmental factors upon the variation of microorganisms, especially upon the phenomenon frequently termed dissociation, has been widely studied (Braun, 1947a). Among the agents used, those which originate from the host environment have created special interest; studies on the *in vitro* effect of such agents may permit a better understanding of the conditions which affect the bacterial population within the host environment. Investigations on the variational aspects of *Staphylococcus aureus* by Nutini and Lynch (1946) have previously demonstrated significant *in vitro* as well as *in vivo* effects with tissue extracts of beef brain, spleen, heart, and kidney. The presence of such extracts was reported to enhance the establishment of white R variants. In recent *in vitro* studies at this laboratory, the selective effect of beef brain extract upon variants of *Staphylococcus aureus* was reinvestigated and the extract's enhancing effect upon the establishment of nonpigmented variants was confirmed.

In contrast to this selective effect of brain extract favoring the establishment of nonsmooth variants, a specific selective effect in favor of the smooth types has been reported when variants of various species were grown in the presence of normal serum (Braun, 1946, 1947b). In work with *Brucella abortus* it has recently been possible to demonstrate that this selective factor is associated with the normal gamma globulin fraction (Braun, 1947b). Therefore, the effect of normal bovine serum and its gamma globulin fraction was tested on *Staphylococcus aureus*. Various strains of hemolytic and coagulase-positive *Staphylococcus aureus*, isolated from the bovine udder, were grown in plain beef extract broth or in beef extract broth containing normal bovine serum, bovine gamma globulin, beef brain extract, as well as a combination of these supplements. Samples from these cultures were plated after various intervals of growth, and the percentage of variant colonies was estimated by counting at least 100 colonies on each test plate.

A summary of the results presented in table 1 demonstrates that (1) the presence of beef brain extract significantly enhances the establishment of non-S variants, (2) the presence of 2 per cent normal beef serum or as little as 25 mg of bovine gamma globulin per 5 ml of broth inhibits the establishment of non-S variants, and (3) the presence of 2 per cent normal serum appears to counteract the enhancing effect of beef brain extract. No differences in growth rates between control cultures and those containing supplements were noted.

The observation on the specific selective effect of the gamma globulin fraction of normal serum upon *Staphylococcus aureus* variants demonstrates that this

activity is not restricted to *Brucella abortus* variants, but may be of a rather general nature.

TABLE 1

Staphylococcus aureus variation in beef extract broth and various added agents

CULTURE MEDIA	AVERAGE % OF VARIANT COLONIES ON PLATES FROM 48-HOUR BROTH CULTURES	AVERAGE % OF VARIANT COLONIES ON PLATES FROM 7-DAY-OLD BROTH CULTURES	TOTAL NUMBER OF TESTS
Beef extract broth..........................	4.08	14.00	86
Beef extract broth plus 0.8–2.0% brain extract...................................	12.75	36.70	108
Beef extract broth plus 2% normal bovine serum..................................	0	0	54
Beef extract broth plus 0.8–2.0% brain extract, 2% normal bovine serum.....	0.57	2.31	60
Beef extract broth plus normal bovine gamma globulin 25–50 mg	0	1.78	32

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NOTE

THERMOPHILIC FERMENTATION OF CELLULOSE IN WOOD

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Research has been resumed on the thermophilic fermentation of cellulose in wood, which prior to 1941 had been conducted co-operatively between the Forest Products Laboratory and the Departments of Agricultural Bacteriology and Biochemistry of the University of Wisconsin (Olson, Peterson, and Sherrard: Ind. Eng. Chem., **29**, 1026; Fontaine, Peterson, McCoy, Johnson, and Ritter: J. Bact., **43**, 701; Fontaine: Summaries of Doctoral Dissertations, Univ. Wisconsin, **6**, 28; Olson: *ibid.*, **7**, 191).

Among the cultures now employed is one obtained from Dr. A. I. Virtanen, Helsinki, Finland, through the kindness of Professor W. H. Peterson. In current work Virtanen's culture produced a mixture of acetic, butyric, and lactic acids in yields of 14.6 per cent of raw sweetgum wood fermented, and 27.0 per cent of the wood previously given a mild acid treatment for slightly loosening the association between the lignin and the cellulosic material.

Thus far the yield of conversion products from raw sweetgum reported herein is lower than those from raw birch (21.2 per cent) and from raw aspen (27.1 per cent) reported by Virtanen (Suomen Kemistilehti, B, **19**, 4). The yield of conversion products from the mildly hydrolyzed sweetgum, however, exceeds that from raw birch and approximates that from raw aspen reported by Virtanen.

NONSPECIFIC ANTISTREPTOLYSIN REACTIONS AND SERUM (OR PLEURAL-EXUDATE) CHOLESTEROL[1,2]

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Hewitt and Todd (1939) occasionally noticed an obviously nonspecific increase in the antistreptolysin titer (AST) in sera that had been contaminated with certain bacteria. Independently, Löfgren (1944) has reported on the same phenomenon.

In comprehensive AST studies on clinical material, Westergren several years ago paid attention to the common occurrence of unexpected rises in AST, often to exceedingly high levels, in sera from hepatitis patients. Kalbak (1947) has had similar experiences. Recently, Sievers (1947), as well as Westergren (1948), has compared the occurrence of these high titers with the results of various liver function tests.

Systematic AST determinations in pleural exudates from patients treated at the Tuberculosis Department of St. Göran's Hospital have shown a high rate of elevated titers, often above 1,000, and not infrequently above 10,000, units in patients whose serum titers were normal or only moderately increased. In most of these cases no definite connection with streptococcal infections could be demonstrated.

When the conditions responsible for these apparently nonspecific AST reactions were studied, it was found that sera and exudates when treated with acid or alkali (and subsequently neutralized) showed striking increases in AST (tables 1 and 6).

At the first glance it seemed very unlikely that, in these four instances of nonspecific antistreptolysin reactions—viz., (1) sera (exudates) contaminated with certain bacteria, (2) a great number of hepatitis sera, (3) pleural exudates, and (4) acid- or alkali-treated sera (exudates)—there could possibly be a common causative factor. Yet from a biological point of view it would be far more logical if those findings were attributable to one and the same basic mechanism.

In the present paper a report is given on a series of experiments conducted in order to arrive at a uniform explanation of the foregoing nonspecific increases in the antistreptolysin titer.

For a better understanding of the problems involved an outline of the mechanism of the streptolysin O hemolysis will have to be drawn according to our

present knowledge. Since the pneumococcal hemolysin in all essentials appears to behave like the streptolysin O, the experiences gained with the former may, *mutatis mutandis*, be utilized in filling up the picture. The red cells are considered to have a mosaiclike membrane of thread-shaped protein and interspersed lipid micelles (Erickson *et al.*, 1938). Cohen *et al.* (1937, 1940) have found that the lytic activity of pneumolysin is associated with at least two functional groupings, one reversibly oxidizable thiol grouping, and the other more or less specific for a certain sterol grouping and configuration. The observations so far made seem to indicate that prior to hemolysis the red cell sterol combines with the lysin (Cohen and Shwachman, 1937; Cohen *et al.*, 1942). The state of oxidation or reduction of the thiol grouping of the lysin conditions its reactivity with the sterol, and thus its adsorption to the red cells (Cohen *et al.*, 1937). Though no direct proof is as yet available, it seems possible that the same thiol grouping provides also the site for attachment of the specific antistreptolysin. If this

TABLE 1

The effect of acid or alkali treatment upon AST

AST		
IN SERUM B 246 TREATED 18 HOURS AT 56 C WITH		
Normality	HCl	NaOH
1/125	8,000	8,000
1/5	4,000	5,000
1/20	16	1,800
1/80	≤16	320
1/320	×	80
1/1,280	×	40
Normal saline	28	

assumption holds true, the antibody blocking of this grouping will, in a way similar to oxidation, prevent the attachment of the lysin molecule to the red cell sterol.

MATERIALS AND METHODS

Materials. Sera were heated for 30 minutes at 56 C. When not immediately examined they were stored at −16 to −20 C. Exudates obtained by thoracocentesis were centrifuged and the clear supernatants treated as the sera.

Antistreptolysin titration. Principally, the AST determinations were made according to Ipsen's (1944) technique. Sheep blood, however, was used instead of rabbit's blood. Todd's concentrated horse immune serum, or patients' sera, the titers of which had been determined by repeated titrations against Todd's serum, were used as standard sera. The calculation of the titers differed somewhat from that applied by Ipsen. A 50 per cent hemolysis end point was fixed for each serum (by direct observation or by interpolation) and compared with the 50 per cent hemolysis end point of the standard serum. Such a manner of proceeding simplifies the calculations, and is at the same time the more correct one

from a theoretical point of view, as shown by, among others, Herbert (1941). The method is described more in detail in a paper on antistaphylolysin determinations (Packalén and Bergqvist, 1947).

Specific absorption. Serum (or exudate) was diluted 1:50 with undiluted, active streptolysin broth. The mixture was incubated in a water bath for 15 minutes at 37 C, and subsequently heated for 30 minutes at 56 C. The effect of the absorption was determined by a routine AST titration in the inactivated mixture. Control absorption with heat-inactivated streptolysin broth or sterile broth showed no reduction of the serum titer.

Precursory adsorption of streptolysin to red cells. Precursory adsorption of streptolysin to the red cells was carried out by mixing both reagents, cooled down to 0 C, in the same proportions as used in ordinary AST determinations and keeping the mixture for 45 minutes in the ice bath. To cooled tubes containing serial dilutions of serum (or exudate) red-cell-streptolysin mixture was then added in amounts to make up the usual proportions of reagents. All transfers were made with cooled pipettes and in cooled tubes and bottles. The specific combination between serum (exudate) antibody and streptolysin, adsorbed in advance to the erythrocyte membrane, was allowed to go on for 45 minutes at 0 C; after which time all tubes were transferred for 15 minutes to a water bath of 37 C. Readings were made in the ordinary way.

Electrophoretic analysis. Electrophoretic fractionation of exudates and sera has been performed by Dr. B. Olhagen[*] according to Tiselius' method (1937). In order to obtain the purest possible albumin or γ-globulin, a compensatory hydrostatic pressure was applied in such a way that the boundary of either of these fractions was kept stationary for about 8 hours (Olhagen, 1945). After the dilution of the electrophoretic fractions was adjusted so that their protein content (determined by the micro-Kjeldahl method) became equal to that of the original dialyzed serum or exudate, their AST was determined in the ordinary way.

Cholesterol determinations. The content of total and esterified cholesterol in sera and exudates was determined photocolorimetrically according to a method described by Swedin (1946). For a part of the determinations a slight modification of this was used. Lipid-extracted sera and exudates were prepared according to Blix's (1941) acetone-ether method in the cold or McFarlane's (1942) ether extraction by freezing below −25 C.

RESULTS

The effect upon AST of specific absorption of sera and exudates, or of precursory adsorption of streptolysin to the erythrocytes at 0 C. The key function of the erythrocyte-membrane-sterol in the mechanism of hemolysis suggests that a nonspecific antistreptolysin reaction might be due to the presence of an excess of reactive cholesterol in serum or exudate. The active groupings of this cholesterol intercept the streptolysin molecules, thus preventing the combination of the

[*] The author is greatly indebted to Dr. Olhagen for his valuable co-operation.

streptolysin with the reacting sterol groupings in the erythrocyte membrane, which necessarily precedes hemolysis.   If this reasoning holds true, prevention of the nonspecific reaction ought to be possible by allowing the streptolysin to combine with the red cells prior to the addition of the serum or exudate rich in cholesterol.   Under ordinary experimental conditions, the red cells, when brought into contact with streptolysin, are all too quickly hemolyzed.   Following adsorption to the erythrocytes at 0 C, however, there is no immediate hemolysis (Herbert and Todd, 1941).   Under these circumstances serum antibody, even though added after the streptolysin, might be afforded an opportunity of specifically inhibiting the lysis that is otherwise certain to take place as soon as the temperature of the mixture is allowed to rise.

TABLE 2

The effect of absorption with streptolysin, and precursory adsorption to erythrocytes, at 0 C, respectively, upon AST in sera and pleural exudates

SERUM	AST			PLEURAL EXUDATE	AST		
	Before	After	After adsorption to r b.c. at 0 C		Before	After	After adsorption to r.b.c. at 0 C
	Absorption with streptolysin				Absorption with streptolysin		
A 9931	100	≦18	25	A 9935	16,000	14,000	≦18
A 5667	160	25	36	A 5679	10,000	9,000	25
A 5833	140	≦16	32	A 5866	4,500	4,000	32
A 9865	700	90	180	A 9867	2,800	2,800	125
A 9822	180	≦16	56	A 9748	1,600	1,000	≦18
A 5024	360	22	90	A 4839	1,400	1,400	64
A 9874	250	≦18	50	A 9871	560	560	36
A 6262	320	≦16	64	A 6266	180	22	32
A 9844	100	≦16	32	A 9835	100	32	32
A 9944	100	25	25	A 9950	80	≦18	25

A series of absorption and adsorption experiments have been conducted in order to demonstrate the correctness of the foregoing conception.   Typical records are presented in tables 2 to 4. As will be seen from table 2, absorption in serum with streptolysin always results in a more or less complete neutralization of the antibody inhibition.   This occurred even in sera with considerably elevated AST values: A 9865, A 5024, and A 6262.   The same was observed in exudates the (low) AST of which were in keeping with the corresponding serum titers.

After precursory adsorption of streptolysin to the red cells, the serum antistreptolysin as a rule showed titers that were only ⅓ to ¼ of their initial values. It is thus obvious that the serum antibodies were not capable of completely preventing the streptolysin already attached to the erythrocytes from exerting its lytic action.   Nevertheless, the remaining inhibitory effect usually exceeded that remaining after specific absorption.

Quite different conditions were encountered in pleural exudates, the AST of which are considerably above the serum titer level.   Specific absorption did not

bring about any noticeable reduction of their titers.   On the other hand, pre-
cursory adsorption of streptolysin to the red cells, as a rule, resulted in a practi-
cally complete disappearance of the strong inhibitory power of these exudates.
Partial exceptions were exudates 9867 and 4839.   However, in the corresponding
sera, A 9865 and A 5024 (both showing specifically elevated titers), a remaining
inhibition was also observed of approximately the same magnitude.   Conse-
quently, there is ample reason to assume that a part of the titer elevation in
these exudates was due to the presence of antibody.   This will explain why the
inhibition in these cases could not be entirely blocked by precursory adsorption.

Corresponding observations were made with hepatitis sera (table 3).   In the
majority of these, specific absorption did not bring about any titer reduction,

TABLE 3

*The effect of absorption with streptolysin, and precursory adsorption to erythrocytes, at 0 C,
respectively, upon AST in hepatitis sera*

PATIENT	HEPATITIS SERUM	AST			
		Before	After	After adsorption to r.b.c. at 0 C	Before or after the icteric stage
		Absorption with streptolysin			
G.M.E.	B 863	3,600	5,000	80	110–125
	B 1626	5,000	3,600	40	
E.A.S.G.	A 9444	9,000	8,000	≦16	
	A 9461	4,000	4,000	32	32–70
	A 9467	400	250	16	
S.A.E.Å.	A 9463	1,600	250	45	250–280
A.M.E.M.	A 9014	1,000	500	360	280–360
E.G.M.J.	A 8894	25,000	500	250	360–900

whereas precursory adsorption of the streptolysin to the red cells resulted in a
more or less complete blocking of the inhibition.   Some hepatitis sera (A 9463,
A 9014, and A 8894), however, behaved differently, which was probably due to
the presence of an increased amount of specific antibody, as indicated by the
elevated AST values observed before or after the icteric stage.

Sera and exudates, the AST of which had increased owing to bacterial contami-
nation or treatment with acid or alkali, behaved on the whole according to the
general rule established above, as did the cholesterol suspension tested (table 4).

Electrophoretic fractionation of serum or exudate with high AST.   Blix, Tiselius,
and Svensson (1941) have shown that the bulk of the serum cholesterol migrates
in an electric field with the β- and α-globulins, the γ-globulin and the albumin
fractions carrying only small portions of the lipid.   On the other hand, the
electrophoretic mobility of most antibodies is that of γ-globulin.

In order to elucidate further the nature of the serum (or exudate) factor giving

TABLE 4

The effect of absorption with streptolysin, and precursory adsorption to erythrocytes, respectively, upon the elevated AST in contaminated or HCl-treated sera (exudate)

SPECIMEN	TREATMENT	AST		After adsorption to r.b.c. at 0 C
		Before	After	
		Absorption with streptolysin		
Serum				
T.P.	Sterile	70	≤ 16	32
T.P.	7^{ds} P. fluor.	4,500	4,000	64
T.P.	7^{ds} P. aerug.	1,800	2,800	45
Serum				
B 246	Sterile	90	25	≤ 18
B 246	7^{ds} P. fluor.	18,000	18,000	≤ 18
B 246	7^{ds} P. aerug.	4,000	4,500	≤ 18
Serum				
B 1043	Sterile	80	20	40
B 1043	HCl	3,600	2,500	160
Exudate				
B 59	Sterile	80	20	40
B 59	HCl	1,000	900	80
Cholesterol susp. 1%		14,000	18,000	≤ 18

TABLE 5

AST in electrophoretically separated fractions of exudate or serum

SPECIMEN	EXP.	AST							
		Whole dialyz.	Electrophoretically separated fractions						
			+ Top	+ Upper	+ Lower	Bottom	− Lower	− Upper	− Top
			−	alb	$alb+\alpha(+\beta)$	$alb+\alpha+\beta$	$(alb+)\alpha+\beta+\gamma$	$\alpha+\beta+\gamma$	$\beta+\gamma$
Exudate A 9747	I	2,000		360	2,000	2,000	2,200	2,000	1,400
			$alb+\alpha$	$alb+\alpha(+\beta)$	$alb+\alpha+\beta(+\gamma)$	$(alb+)\alpha+\beta+\gamma$	$\beta+\gamma$	γ	−
Exudate A 9747	II	4,000	2,000	4,000	2,800	2,800	2,000	500	
A 9761		4,500	4,500	8,000	4,000	4,500	4,000	200	
Serum B 1679		3,200	≤ 40	110	640	2,500	5,000	$\geq 20,000$	

nonspecific AST increases, a few exudates with high titers and, for comparison, a serum from a scarlet-fever patient, were subjected to electrophoretic separation.

The results are shown in table 5. In the exudates it is seen that the AST of the end fractions had decreased more or less, most clearly so when they were obtained in a comparatively pure state by applying a compensating hydrostatic pressure to the fluid in the electrophoretic cell system. In experiment I the albumin fraction, and in experiment II the γ-globulin fraction, had been made electrophoretically homogeneous by this procedure. The serum from the scarlet-fever patient behaved quite differently in the electrophoresis experiment. The

TABLE 6

The effect of lipid extraction upon AST in sera and exudates

SPECIMEN		AST			
	Before extraction		After extr. acc. to		In serum
		Blix	McFarlane		
			−78 C	+20 C	
Exudates					
A 9907	28,000		≤18	14,000	64
A 9935	11,000	40			64
A 9747	8,000	125			500
A 9905	2,500		40	1,250	90
A 9920	2,200	640			560
A 8299	1,000	450			1,000
A 9949	900		140	4,500	180
A 8726	800	200			500
A 9950	110	32			64
B 59	90	40			56
Sera (hepatitis)					
A 9825	5,000		500	5,000	
B 4063	320,000		80	56,000	
Serum (scarlatina)					
Epid. S.E.	900		560	640	
Serum (normal)					
A 8470	80		80	80	

highest AST value was observed in the γ-globulin fraction. With increasing electrophoretic mobility of the fractions their AST gradually decreased.

AST in sera and exudates after lipid extraction. The lipids may be more or less completely removed from sera (or exudates) without noticeably impairing the proteins by Blix's (1941) acetone-ether extraction method at low temperatures or by McFarlane's (1942) ether extraction at temperatures below −25 C. The changes in AST after lipid extraction according to either of these methods have been studied in several exudates and sera. Typical results are given in tables 6 and 7. It will be seen that pleural exudates and hepatitis sera with elevated AST after lipid extraction as a rule show an almost complete loss of their in-

hibitory power. Exceptions to this rule sometimes may be noted, e.g., in exudates A 9747, A 9940, A 8299, and A 8726. These exudates, however, derived from patients whose sera also had an increased AST, which indicates that the

TABLE 7

The effect of contamination and treatment with acid or alkali upon AST in lipid-extracted exudates and sera

SPECIMEN	AST											
	Before extraction				After extraction							
	Sterile	Infected	Treated with		Sterile		Infected		Treated with			
									HCl		NaOH	
			HCl	NaOH	(a)*	(b)†	(a)	(b)	(a)	(b)	(a)	(b)
Exudates												
A 9907	28,000		22,000	32,000		≤20		≤20		64		≤16
A 9935	11,000				40		40		≤16		≤16	
A 9950	110	25,000	9,000	2,500	50		100		≤16		≤16	
B 59	90	1,800	4,500	3,200	40	≤18	40			≤18		≤18
Sera												
A 8470	125	7,000	10,000	10,000		80		80		≤25		≤25
B 147	220	25,000	1,800	2,800	80	≤18	80			≤18		≤18

* (a) Extracted according to Blix's method.
† (b) Extracted according to McFarlane's method.

TABLE 8

AST in relation to cholesterol changes developing in exudates and sera infected with Pseudomonas fluorescens

SPECIMEN	AST		CHOLESTEROL		
	Titer	Increase	Total	"Free"	"Free"/Total
			mg %	*mg %*	*per cent*
Exudate					
B 59 sterile	90		98	41	42
B 59 infected	1,800	20×	101	43	42
Serum					
A 8470 sterile	125		139	69	50
A 8470 infected	7,000	56×	149	89	60
Serum					
B 147 sterile	220		225	71	32
B 147 infected	25,000	110×	243	147	60

rise was probably due to the presence of specific antistreptolysin. For the extractions performed with specific antisera, such as the scarlet-fever-patient serum (Epid. S.E.), demonstrated that antibody is not removed together with

the lipid fraction.   In table 6, attention should be given also to the fact that extraction with ether at room temperature did not result in any conspicuous reduction of the titer, though extraction below -25 C did.

If exudates or sera initially showing strong nonspecific rises in AST, or anyway capable of showing such rises if contaminated or treated with acid or alkali, had been subjected to lipid extraction, no further titer increase could be brought about by the above-mentioned procedures (table 7).

AST and amount of total and "free" serum (or exudate) cholesterol.   Numerous investigators (Boyd and Connell, 1938; Epstein and Greenspan, 1936; Greene *et al.*, 1940; Thannhauser and Schaber, 1926) have shown that in hepatitis sera there is a striking decrease in ester cholesterol, the total cholesterol level remaining unchanged.   The possibility was therefore taken into consideration that the clue to the origin of the nonspecific rise in AST in hepatitis sera might be found

TABLE 9

AST in relation to cholesterol changes developing in sera treated with HCl or NaOH

SERUM	TREATED WITH	AST	CHOLESTEROL		
			Total	'Free"	"Free"/Total
			mg %	*mg %*	*per cent*
A 8470	NaCl	125	165	60	36
	HCl	10,000	142	55	39
	NaOH	10,000	135	73	54
B 594	NaCl	250	172	74	43
	HCl	7,000	146	42	29
	NaOH	18,000	151	79	52
B 1043	NaCl	640	270	161	60
	HCl	40,000	262	128	49
	NaOH	28,000	250	176	70

in the ensuing increase in "free" serum cholesterol.   If so, then corresponding changes in the condition of serum and exudate lipids might by analogy be expected also in other instances of nonspecific AST.   For more exact information on this point, a number of cholesterol analyses have been made with sera and exudates.   Representative records are given in the following tables:

It was found (table 8) that a 20-fold increase in AST in exudate B 59 after 7 days' incubation (at room temperature) with *Pseudomonas fluorescens* was not associated with any change in the proportions of "free" and ester cholesterol. In serum A 8470, which showed a 56-fold rise in titer after infection, a shift by 10 per cent (from 50 to 60 per cent) from bound to "free" cholesterol was noted, and in serum B 147, where the titer rose 110-fold, the "free" cholesterol had increased from 32 to 60 per cent.   Hence, the increase in "free" cholesterol observed in sera A 8470 and, especially, B 147 might possibly have some connection with their rise in AST.   However, the absence of such an increase in "free" cholesterol as regards exudate B 59 indicates that the primary cause of

these AST rises apparently is to be sought in some mechanism other than the hydrolysis of ester cholesterol.

This inference is corroborated by the cholesterol analysis of sera treated with 2 N HCl at 56 C for 18 hours (table 9). A slight decrease in the total cholesterol content was noted as the result of this energetic chemical treatment. The amount of "free" cholesterol decreased to the same, or to a still higher, degree. On the other hand, alkali treatment (2 N NaOH at 56 C for 18 hours) did result in a hydrolysis of ester cholesterol. This may, but by no means must, imply that the rise in AST in these sera was due to the increased content of "free" cholesterol.

TABLE 10

AST and cholesterol in pleural exudates

PLEURAL EXUDATE	AST	CHOLESTEROL			PLEURAL EXUDATE	AST	CHOLESTEROL		
		Total	"Free"	"Free"/ Total			Total	"Free"	"Free"/ Total
		mg %	*mg %*	*per cent*			*mg %*	*mg %*	*per cent*
A 8282	70	115	69	60	A 9809	400	64	45	70
B 59	90	108	56	52	A 8338	560	98	64	65
A 7839	100	102	47	46	A 9871	560	146	71	49
A 8105	110	72	34	47	A 9793	640	107	66	62
A 9950	110	101	61	60	A 8726	800	78	36	46
A 8571	140	45	8	18	A 8299	1,100	86	33	38
A 8038	180	92	51	55	A 9775	1,100	128	68	53
A 7871	220	38	19	50	A 9930	1,600	132	38	29
A 9949	220	74	54	73	A 9850	2,500	68	36	53
A 9905	250	87	51	59	A 9907	2,500	74	45	61
A 9803	360	62	43	69	A 9867	2,800	138	83	60
A 8098	400	88	47	53	A 9890	2,800	186	102	55

The cholesterol analyses of pleural exudates (table 10) reveal that no correlation whatever obtains between their AST and their total cholesterol content, nor their levels of "free" and ester cholesterol, respectively.

AST in pleural exudates and the duration of the effusion. When the clinical data pertaining to the pleurisy patients was compiled, an obvious relationship emerged between AST and the duration of the pleural effusion. This is illustrated by figure 1, in which the AST values of 282 exudates from 146 patients are plotted against the duration of their effusions. It will be seen that the AST during the first month of pleural exudation as a rule remained "normal," the average titer being 100 units. Only in 12 cases did the exudate titer exceed 200 units. In 11 of them the serum titer was within 50 per cent of the exudate value. During the following months the titers continuously increased until reaching an average titer of 1,400 units during the fifth month. A considerable scatter was observed of the individual AST values with increasing persistence of the exudate. After the fifth month of exudation, the average titer remained about the same. Possibly there was a slight tendency toward a further increase in titer in cases

whose exudate persisted for years.   For comparison a curve representing average serum titers for the same patients is inserted in the figure.   It will be seen that the sera show only insignificant increase from approximately 100 units in the first half-year to 140 units subsequently.   It should be mentioned that the idiopathic exudates did not differ in any respect from those developing after institution of artificial pneumothorax, or after pneumonolysis.

Finally, it was obvious that the AST increase in the exudates very often coincided with certain characteristic changes in their cellular content.   Although
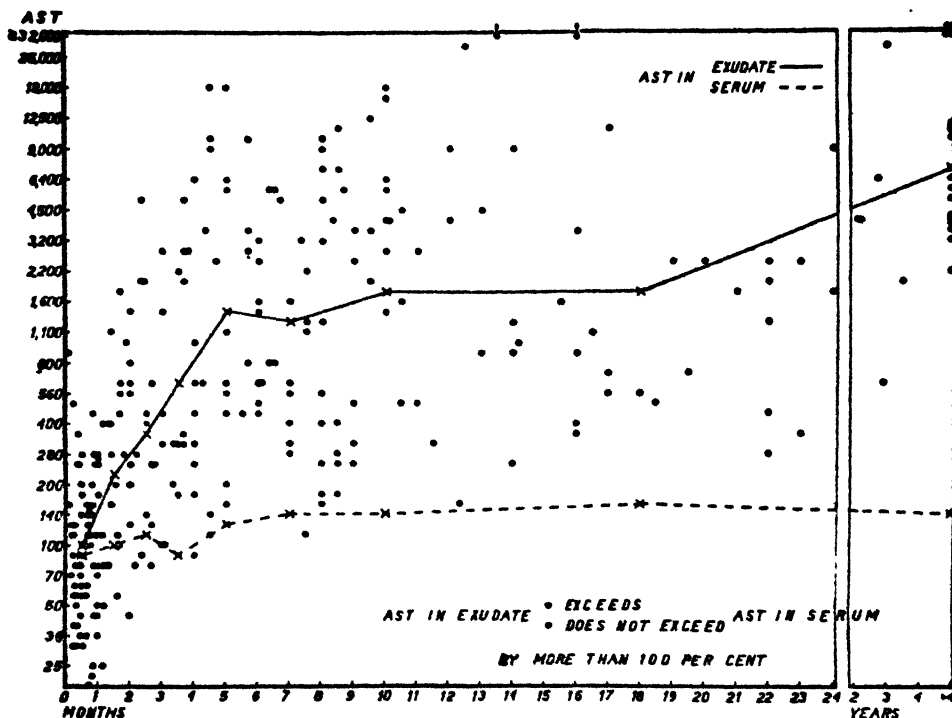
Figure 1. AST in pleural exudates and duration of exudation.

lymphocytes predominated in the fresh exudate, increasing numbers of polynuclear leucocytes entered into the picture when the exudate had persisted for some time, the AST commencing to rise.   Unfortunately, detailed figures cannot be presented, as this statement is based on routine examinations, in which the numbers of lymphocytes and leucocytes were recorded only as rough estimates, i.e., "a few," "moderately," "abundantly," or "predominantly."

DISCUSSION

The supposed nonspecific nature of AST rises (1) in sera and pleural exudates after contamination with certain bacteria, (2) after treatment with acid or alkali, (3) in most of the hepatitis sera, and (4) in pleural exudates of some mini-

mum duration has been confirmed, *inter alia*, by the nonabsorbability of the inhibitory factor with specific streptolysin, as well as by the blocking of the inhibition through precursory adsorption of streptolysin to the red cells at 0 C.

In their study of the nonspecific inhibition following bacterial contamination, Hewitt and Todd (1939) found the neutralizing factor to be present in the acetone-soluble fraction of the serum. They assumed the bacteria to "effect some change in the condition of serum lipins, enabling them to neutralize streptolysin O." Since cholesterol was found to inhibit streptolysin hemolysis in high dilutions, the authors suggested that the bacteria liberated cholesterol by their proteolytic action on the serum proteins. However, the mechanism of this "liberation," and consequently the entire problem of the role actually played by the cholesterol in the inhibition of streptolysin hemolysis, remained obscure.

The observation that electrophoresis reduced the nonspecific inhibitory power just in those fractions, viz., the albumin and γ-globulin portions, in which according to Blix *et al.* (1941) the cholesterol content is also reduced seems to be strong evidence in favor of the cholesterol actually being responsible for the inhibition in question. The removability of such an inhibitory power, either actual or potential, by the aid of lipid extraction is then quite a logical sequence.

A hypothesis that an increase in "free," i.e., not esterified, cholesterol, such as observed in hepatitis sera, might be considered the real clue to most, if not all, nonspecific AST rises in sera and exudates could not be confirmed by actual analyses of their "free" and esterified cholesterol content. Only in alkali-treated sera (and exudates) was a hydrolysis of cholesterol ester consistently demonstrated. Possibly the increase in "free" cholesterol observed in some sera after contamination may have contributed to the particularly pronounced rise in AST exhibited by them. Since, however, in other contaminated sera with raised AST the "free" cholesterol level remained unaltered, it is unlikely that the *primary* increase in these sera was attributable to such a change in the condition of their serum cholesterol. If the conception of serum (exudate) cholesterol as the primary factor causing nonspecific AST reactions is still to be maintained, another explanation has to be found of the actual mechanism.

According to present opinion, the bulk of the serum lipids occur as lipoproteins having the character of secondary valence complexes which are held together by forces of the van der Waals type (Chargaff, 1944). The bonds between the lipids and the serum proteins appear in the main to be weak ones (Chargaff, 1944). Under such circumstances the most promising avenue of approach to the problem seems to be the following hypothesis: As long as these lipoprotein complexes are unimpaired, the active OH groups of the cholesterol remain engaged in the linkage to the protein and are not available for the attachment of the streptolysin molecules. However, as soon as the complex is broken up—whether by the action of acid or alkali, bacterial enzymes or enzymes liberated or activated through the disintegration of leucocytes in exudates of long duration, or by the decay of liver cells in hepatitis—the hydroxy groups of the cholesterol molecules are uncovered, and a combination with the sterolaffine groups of the streptolysin is rendered possible. This combination implies a blocking of the necessary

attachment of the streptolysin molecules to the red cell membrane sterols. It should be stressed here that in hepatitis another mechanism is also conceivable: the dysfunction of the liver cells may primarily result in an incomplete synthesis of lipoprotein complexes. But the final outcome is the same, viz., cholesterol molecules with free hydroxy groups in the serum.

The chemical methods available at the present moment for quantitative estimation of the proportion of protein-linked and actually free cholesterol in serum are as yet not quite appropriate, but it is to be hoped that further progress in this field will afford us more conclusive observations concerning our problem.

SUMMARY

Strong increases in antistreptolysin titer, AST, independent of specific stimuli occur (1) in sera (and pleural exudates) contaminated with certain bacteria, or (2) in sera treated with acid or alkali, (3) in most hepatitis sera, and (4) in pleural exudates of some duration.

The nonspecific nature of these rises was confirmed by the nonabsorbability of the inhibitory factor with specific streptolysin and the blocking of the inhibition by precursory adsorption of streptolysin to the red cells at 0 C.

In electrophoretic separation experiments the inhibitory factor was found to remain chiefly in the β- and α-globulin fractions, i.e., just those fractions in which the serum cholesterol accumulates, whereas the antibodies are found in the γ-globulin fraction.

Lipid extraction of sera and exudates abolished nonspecific AST reactions in them, and rendered them simultaneously unable to respond with a rise in AST to bacterial contamination or treatment with acid or alkali. Specific antistreptolysin, on the other hand, remained unaffected by this procedure.

It has not been possible to prove, by cholesterol analyses, that an increase of "free," i.e., not esterified cholesterol—as seen in hepatitis sera—should be considered the consistent explanation of most nonspecific rises in AST. However, the hydrolysis of ester cholesterol may play some part in the origination.

It is suggested that the primary mechanism of the nonspecific AST reactions is a breaking up of the lipoprotein complexes, in which the serum lipids are supposed to occur. The uncovered, active OH groups of the freed cholesterol combine with the streptolysin molecules, thus preventing them from attacking the red cells. The breaking-up process may be brought about, *inter alia*, by the action of acid or alkali, or bacterial enzymes, or enzymes liberated or activated through the disintegration of liver cells in hepatitis, or leucocytes present in exudates of some duration.

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A METHOD FOR INVESTIGATING LARGE MICROBIAL POPULATIONS FOR ANTIBIOTIC ACTIVITY[1]

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In their search for new antibiotics, investigators throughout the world have isolated from nature and tested thousands of microbial strains. Nevertheless only a few useful antibiotics have been found and, from this, it is obvious that only a minute fraction of the microflora of nature produce desirable antibiotics. A method that would drastically increase the number of strains screened without proportionate increase in labor would improve the chances of finding new antibiotics. It would also be useful for the selection of high-yielding variants in a pure culture of an antibiotic producer.

The most serious drawback of earlier methods for testing large populations was that they detected only certain definite types of antagonists. Thus the "crowded plate" method (Waksman, 1945) detected only soil organisms inhibiting other soil organisms and producing an antibiotic after not more than one or two days of incubation. Foster and Woodruff's method (1946) detected only antagonists that produced antibiotics on media supporting the growth of saprophytic mycobacteria and that inhibited such bacteria. The development (Williston *et al.*, 1947) of various media on which both pathogenic tubercle bacilli and antagonists will grow further extends the usefulness of Foster and Woodruff's method (1946) but does not remove its fundamental limitations.

Stansly (1947) and Wilska (1947) described methods for spraying sensitive bacteria onto plates on which colonies of soil organisms had grown for several days. It was thus possible to test antibiotic action against a greater variety of bacteria than was possible before. Spray methods are still, however, restricted to media on which both antagonistic colonies and sensitive bacteria can grow. In addition, there is the ever-present possibility that inhibition zones would be caused by the exhaustion of nutrients around a colony, or by an inhibitory pH, rather than by true antibiotic production. The latter objection applies also to methods that add test bacteria by flooding the surface of the plate with a liquid suspension of sensitive organisms.

Fleming (1942) suggested coating a plate on which an antagonist has grown with a layer of sterile agar, on which a sensitive bacterium could be streaked. He sought to test individual isolates, and did not describe methods for testing large populations. With Fleming's agar layer suggestion as a basis, a method has been developed whereby large populations can be tested for many types of

antagonists capable of inhibiting various microorganisms, or even antagonists inhibiting nonmicrobial organisms.

EXPERIMENTAL PROCEDURES

The material serving as the source of antagonists is plated on agar so as to have 30 to 50 evenly distributed isolated colonies. After suitable incubation the colonies are covered with a layer of sterile agar, followed by a layer of agar containing the organism whose inhibition is to be tested. After further incubation clear zones over certain colonies disclose the antagonists. The addition of agar layers is a technique remarkably well adapted to replication, and if each step in the procedure is made simple enough, 200 to 1,000 plates can be prepared in a few hours by two workers. From 6,000 to 50,000 colonies are screened in an afternoon.

The agar layers, in the order to which they are added to the plate, are: (1) *Foundation layer:* a layer of about 15 ml sterile agar medium, of a composition suitable for the growth of the antagonist; e.g., peptone beef extract, corn meal, Czapek, wort agar, etc. (2) *Seeding layer:* a layer of 0.5 ml soft agar medium (0.25 per cent agar), in which is diluted the microflora to be tested. The dilution is calculated to give 30 to 50 colonies per plate. The layer of soft agar soon dries down to such a thinness that all colonies are surface colonies. If it is not necessary to have all the colonies absolutely on the surface, 5 ml of a nutrient agar containing 1.5 per cent agar can be substituted for the softer medium; this is even better adapted for mass replication. (3) *Diffusion, or barrier, layer:* 5 to 10 ml sterile agar of special composition, whose use is illustrated in problem 3. (4) *Test layer:* 3 or more ml of agar medium (0.5 to 1.5 per cent agar) containing a suspension of bacteria whose inhibition is to be tested.

The actual working out of the method and its flexibility will be illustrated by describing its use in four diverse problems in antibiotic screening.

Problem 1. To test soil for antagonists against Escherichia coli—the antagonists in the soil to grow on nutrient agar. A suspension of soil in distilled water was centrifuged lightly to remove coarse particles. The supernatant was assayed on nutrient agar for viable cells, then stored at 5 C. The colony count was determined after incubation for 2 to 4 days at 28 C. Foundation layers of 15 ml nutrient agar were added to 200 plates. Since it was not imperative to have the soil colonies strictly surface colonies, a seed layer of 5 ml nutrient agar (1.5 per cent agar) was used. The soil suspension was diluted so that 5 ml contained 50 viable cells. The last step in the dilution was made into 500-ml portions of melted nutrient agar, kept in a 45 C water bath. Five-ml seed layers were pipetted onto the foundation layer, and the plates were then incubated at room temperature for 1 week. There were now 200 plates containing about 50 colonies each, or a total of 10,000 colonies.

The diffusion layer was omitted, and a test layer was added directly over the soil colonies. To prepare the test layer, 20 ml of a 24-hour broth culture of *Escherichia coli* were added to 1,000 ml nutrient crystal violet agar kept in a 45 C water bath. Five ml of this material were pipetted to each of 200 plates to form

the test layer.   The small concentration of crystal violet, 1:750,000, prevented soil fungi, actinomycetes, and gram-positive bacteria from growing up into the test layer, but it did not appreciably inhibit the growth of *E. coli*.   After incubation at 37 C overnight numerous clear zones were found (figure 1).

Problem 2.   To test soil for antagonists against Staphylococcus aureus—the antagonists in the soil to grow on corn meal agar.   A suspension of soil was prepared as described for problem 1 and assayed on corn meal agar.   Use of this medium would promote the growth of colonies that may have been suppressed or overgrown on nutrient agar, and, in addition, a different nutritive medium for antibiotic production would probably reveal antagonists missed on nutrient agar. The foundation and seed-layer medium was corn meal agar.   Twenty ml of a 24-hour broth culture of *S. aureus* were added to 1,000 ml melted nutrient agar at
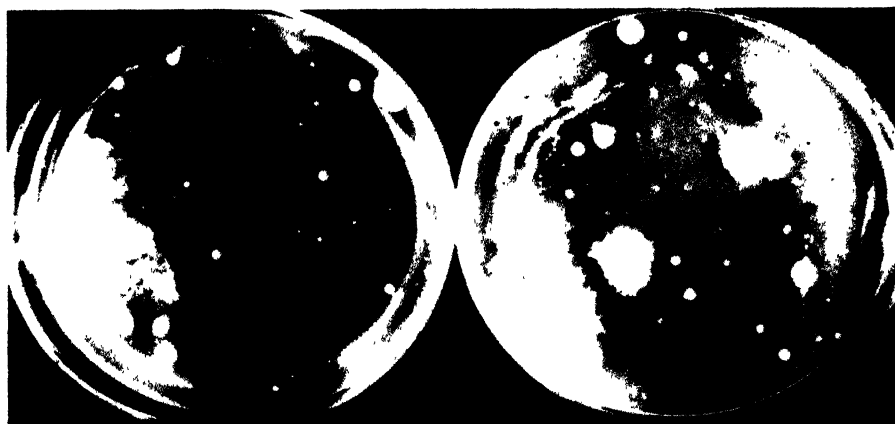
Figure 1. Typical plates of soil organisms growing on nutrient agar and tested by the addition of a test layer of crystal violet nutrient agar containing *Escherichia coli*.   *Left:* Plate showing large inhibition zone.   In most cases the active colony is the one at the mathematical center of the zone.   *Right:* Typical plate with no active colonies.

45 C, and test layers of 5 ml each were added to the plates.   Since crystal violet inhibits *S. aureus*, it could not be used; however, by incubating the test layers for 6 hours instead of overnight, the soil colonies did not grow up into the test layer to any appreciable extent, whereas the *S. aureus* colonies in this period grew sufficiently to make inhibition zones clearly visible.   It was convenient to add test layers in the afternoon, store the plates overnight at 5 C, then incubate at 37 C for 6 to 8 hours.   This experiment illustrates the use of different media in the foundation and test layers.

Problem 3.   To study the variation in antibiotic activity of strains of a pure culture of an actinomycete (A-13) which was a good antibiotic producer.   If the spores of actinomycete A-13 were plated on nutrient agar and tested against *E. coli*, the size of the zones of inhibition around individual colonies could be taken as a measure of the antibiotic activity of the organisms in each colony.   Those colonies with exceptionally large zones of inhibition could be isolated for further study.

A preliminary experiment showed (1) that all colonies had to be on the surface, for even partly buried colonies had distinctly smaller zones than the surface colonies, and (2) when a plate contained more than 2 or 3 colonies the large (60-mm radius) inhibition zones overlapped, making impossible the comparison of zone size.

The use of a 0.5-ml seed layer of soft (0.25 per cent agar) nutrient agar readily ensured surface colonies. The spores were diluted in the melted agar kept at 45 C in a water bath. Aided by tilting and shaking of the plates, the 0.5-ml inoculum spread easily, especially over freshly poured, solidified but still warm foundation layers. Two people working as a team could inoculate 200 to 300 plates in an hour.

The large zones of inhibition caused a more serious difficulty, for unless plates containing 30 to 50 colonies could be tested, the aim of testing large populations was defeated.

Smaller average zone size might be obtained by (1) using a medium less favorable for antibiotic production than nutrient agar, (2) growing all of the colonies subsurface, or (3) testing the colonies after only 2 or 3 days of incubation, before the peak of activity was reached. Such measures were discarded because it was feared that suboptimum conditions for antibiotic production would result in a nonsignificant increase in variability of zone sizes.

The use of organisms more resistant to the antibiotic than *E. coli* might solve the problem. *Serratia marcescens*, *Eberthella typhosa*, *Alcaligenes faecalis*, *Escherichia communior*, and *Bacillus mycoides* were tried, but none gave suitable small zones. Continued research with other bacterial species might have disclosed strains more suitable than those tried. A simple physical method was discovered, however, by which one could at will decrease the zone size to any desired degree. A 10-ml sterile diffusion layer was interposed between the A-13 colonies and the test layer. If the diffusion layer was simple nutrient agar, the additional distance the antibiotic had to diffuse resulted in a decrease in zone size of a few millimeters at most. But incorporation into the diffusion layer of substances that would partially adsorb the antibiotic would result in a sharp decrease in zone size. Several adsorbents were tried, and decolorizing carbon (norit A) was found satisfactory. The zone size varied inversely with the concentration of norit A in the diffusion layer (table 1).

The decrease in zone size varied from experiment to experiment, and in actual practice 0.75 per cent norit A in a 10-ml diffusion layer of nutrient agar gave satisfactorily small zones. Norit A was added to the nutrient agar before autoclaving. Since the heavy powder settled rapidly in melted agar, it was necessary to shake the suspension just before use. The use of a diffusion layer as outlined is capable of widespread application, since most antibiotics can be adsorbed with one substance or another.

The procedure finally adopted for study of this active actinomycete A-13, was as follows: A suspension of conidia of A-13 was filtered six times through absorbent cotton, assayed on nutrient agar, and then stored in the refrigerator until the

assay colonies had grown. To petri dishes were then added foundation layers of nutrient agar, and 0.5 ml of soft (0.25 per cent agar) nutrient agar suspension of conidia calculated to contain 40 viable spores per 0.5 ml. The plates were incubated at 28 C for 5 to 7 days. A diffusion layer of 10 ml nutrient agar containing 0.75 per cent norit A was added, followed by a test layer of 3 ml nutrient agar (0.5 per cent agar) containing 1 to 2 ml of 24-hour broth culture of *E. coli* per 100 ml agar. After incubation overnight at 37 C, zone sizes of the actinomycete colonies were measured.

Problem 4. To test soil for antagonists against a green plant. By substituting small plant seeds for the bacteria in the test layer, the agar overlay method might be used for detecting microorganisms inhibiting (or stimulating, or affecting in another specific way) germinating seeds. The inhibition of germinating seeds by javanicin and oxyjavanicin (Arnstein *et al.*, 1946), polyporin (Bose *et al.*, 1948), and penicillin (Ribeiro, 1946; Smith, 1946) has been reported.

TABLE 1

Effect of norit A on zone size

CONCENTRATION OF NORIT A IN DIFFUSION LAYER	AVERAGE ZONE SIZE (RADIUS)
%	mm
0	54
0.3	25
0.5	18
0.7	16
0.8	13
1.0	8

The several days necessary for seed germination (as compared to the 6- to 18-hour incubation period for bacteria) complicated the method by allowing time for soil organisms to grow up into the test layer; and no simple means for testing large populations was found. Nevertheless, the success obtained by the following procedures illustrates the adaptability of the method.

Sterile filter paper was placed in the bottom of petri dishes, followed by a 20-ml foundation layer of corn meal agar, and a 3-ml seed layer of a soil suspension diluted in corn meal agar to give 40 colonies per plate. The plates were incubated at room temperature for 1 week. Before adding the seeds, the entire agar layer was carefully overturned into a sterile petri dish, so that the underside of the foundation layer was now on top and the soil colonies on the bottom. It helped to use a spatula inserted between the petri dish rim and the agar layer, and under the filter paper. By peeling off the filter paper a clean sterile surface was uncovered on which to add the test layer, with lessened danger of contamination by soil organisms.

Redtop grass seeds (*Agrostis alba*) were surface-sterilized by being soaked in a solution of sodium hypochlorite for 10 minutes, then washed in sterile distilled water. The test layer medium consisted of 7 ml of half-strength Shive solution (Miller, 1931) in melted agar at 45 C, to which had been added about 1 part in 5

of the seeds. The test layer was pipetted onto the foundation layer, and the seeds were spread uniformly over the surface by gentle tilting of the plate. The plates were incubated in diffuse light at room temperature for 3 days. Despite some contamination around the edge of the plates, most of the seeds germinated cleanly. Clear-cut circular zones of inhibition were seen around some soil colonies.

SUMMARY

A general method is described whereby large numbers of microbial colonies from diverse sources can be tested for antibiotic activity. Use of the method is illustrated by descriptions of experiments in which (1) a soil flora is tested for strains producing antibiotics against *Escherichia coli*, or *Staphylococcus aureus;* (2) a pure culture of a highly active actinomycete is tested for variants with increased activity; and (3) a soil flora is tested for strains inhibiting the germination of redtop seeds.

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THE NUTRITIONAL REQUIREMENTS OF TREPONEMATA

I. Arginine, Acetic Acid, Sulfur-Containing Compounds, and Serum Albumin as Essential Growth-promoting Factors for the Reiter Treponeme

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There have been many unsuccessful attempts to cultivate the causative organism of syphilis on both natural and synthetic media since its demonstration by Schaudinn in 1905 (cf. Kast and Kolmer, 1929; Eagle, 1948). Supniewski and Hano (1933) and Scheff (1935) were among the first to approach the problem by studying the metabolism and growth requirements of such treponemata as could be cultivated on artificial media. Their findings, as well as those of Kast and Kolmer (1940), Rosebury and Foley (1942), and Wichelhausen and Wichelhausen (1942), indicated that one or more factors present in body fluids and tissues (e.g., blood serum, ascitic fluid, kidney tissue, liver tissue) were essential for the growth of these organisms. More recently, Little and SubbaRow (1945) and Whiteley and Frazier (1948) reported that the Reiter treponeme, a nonpathogenic organism purporting to have been cultivated from a syphilitic lesion (Reiter, 1926) but serologically related to some of the saprophytic treponemes isolated from the mouth (Robinson and Wichelhausen, 1946; Eagle and Germuth, 1948), could be cultivated in a simplified medium consisting of an acid hydrolyzate of casein (or a mixture of the component amino acids of that protein), supplemented with glucose, several vitamins (ascorbic acid, choline chloride, niacin, calcium pantothenate, pyridoxine, riboflavin, and thiamine), a sulfur-containing reducing substance (sodium formaldehydesulfoxylate or sodium thioglycolate), and serum albumin.

In the present study, also, the nutritional requirements of the cultivable Reiter strain were investigated in the hope that such data might offer clues to the growth requirements of the more fastidious pathogenic *Treponema pallidum*. To date, four substances have been identified as being essential for growth under the conditions of the present experiments: (1) arginine, (2) acetic acid, (3) any one of a series of compounds containing either free sulfhydryl groups or sulfur groups capable of being reduced to the sulfhydryl form and, (4), in confirmation of the data of Little and SubbaRow (1945) and of Whiteley and Frazier (1948), crystalline serum albumin. The latter has been shown to be a substitute for the nondialyzable fraction of whole serum. The phosphate buffer, which was added for its poising action, and ascorbic acid, which was added to maintain anaerobiosis, were apparently not essential nutrients. There remain to be identified

the growth factors present in the minute amounts of yeast extract and casein digest which must be added to the culture fluid for satisfactory growth. The importance of glucose also remains to be determined.

METHODS AND MATERIALS

The components of the culture fluid which was the starting point of the present investigation were (1) Brewer's thioglycolate medium (Brewer, 1940), with the following composition:

Trypticase (an enzymatic digest of casein)	15 g/L
L-Cystine	0.75 g/L
Glucose	5 g/L
Yeast extract	5 g/L
Sodium chloride	2.5 g/L
Sodium thioglycolate	0.5 g/L
Resazurin	1 mg/L
Agar	0.75 g/L

and (2) rabbit or human serum, sterilized by filtration through a glass filter (Corning UF), and heated at 60 to 63 C for 1 to 2 hours. Both were essential, a 9:1 mixture giving luxuriant growth. For the purposes of this study, however, these two components were used in subliminal concentrations which alone did not support the growth of the organisms, presumably because one or more essential ingredients were at less than the threshold concentrations necessary for growth. A variety of substances were then assayed for their ability to permit multiplication of the organisms when added to the otherwise quantitatively inadequate basal medium. These compounds were made up at neutral pH in isotonic concentration, or in solutions adjusted to isotonicity by the addition of sodium chloride. In individual experiments in which they might have complicated the results, cystine, resazurin, and agar were omitted from the basal thioglycolate medium. Similarly, as is indicated in the text and in the tables, washed inocula were used when the presence of the original culture fluid would have complicated the interpretation of the experimental results.

The amino acids, the fatty acids, the phosphate buffer, and the thioglycolate medium were sterilized by autoclaving; ascorbic acid and sulfhydryl compounds were glass-filtered in acid solution and adjusted to pH 6.8 to 7.2 immediately prior to use. The volumes were adjusted with M/7 sodium chloride to a total of 5 ml in 18-by-150-mm pyrex test tubes, and the tubes incubated at 37 C in Brewer anaerobic jars under an atmosphere of hydrogen. Direct microscopic counts (cf. Magnuson, Eagle, and Fleischman, 1948) were made after 4 to 6 days, at the peak of the growth curve.

EXPERIMENTAL RESULTS

Arginine as a growth-promoting factor. A typical experiment that demonstrates the effectiveness of arginine as a growth-promoting factor is summarized in table 1. As is there indicated, 0.2 ml of an actively growing culture on thioglycolate medium enriched with 10 per cent serum were used as the inoculum, this inoculum constituting the thioglycolate portion of the basal medium. This

was supplemented by 0.5 ml of heated rabbit serum, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and cysteine hydrochloride to a final concentration of 1:1,000, all in a total volume of 5 ml. In the absence of arginine the original inoculum of 2 million organisms per ml had disappeared in the course of 6 days. The addition of arginine had a striking effect. The smallest concentration with a significant
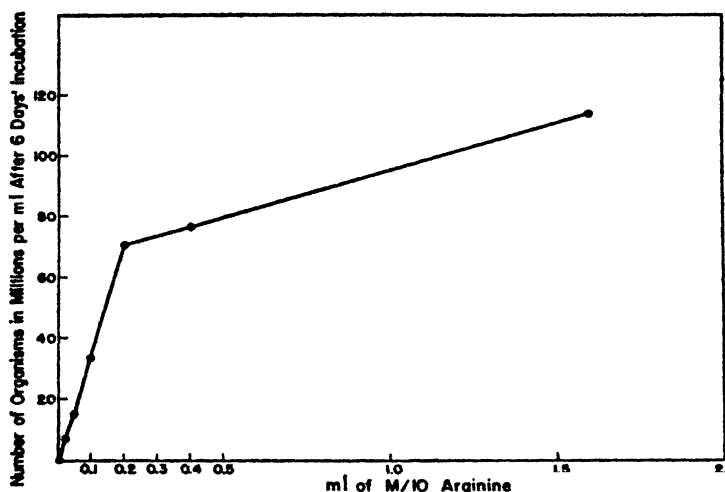

Figure 1. The growth-promoting effect of arginine on the Reiter treponeme as a function of its concentration.

TABLE 1

The growth-promoting effect of arginine on the Reiter treponeme as a function of its concentration

Ml of M/10 arginine in total of 5 ml ...	0	0.025	0.05	0.1	0.2	0.4	1.6
Number of organisms in millions per ml after 6 days' incubation.......	0	7	15	34	71	77	114

Varying amounts of M/10 arginine were added to a basal medium consisting of 0.5 ml heated rabbit serum, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and 0.33 ml of M/10.5 cysteine hydrochloride, in a total volume of 5 ml (including the inoculum of 0.2 ml of an actively growing culture in the serum: thioglycolate medium). This inoculum served to provide those ingredients essential for growth present in the thioglycolate medium. The initial number of organisms was 2 million per ml; the number present after 6 days' incubation at 37 C was determined by direct count.

effect on growth was 0.0005 M; but beyond that level the number of organisms increased in proportion to the concentration of arginine, 6-day cultures reaching a count of 114 million per ml at an arginine concentration of 0.03 M (0.5 per cent). It is apparent that the arginine did not act as a trace factor; and its activity was quantitatively unaffected by recrystallization.

The dependence of the degree of growth on the amount of arginine used is shown graphically in figure 1, which presents the data of table 1. A number of similar experiments with qualitatively similar results are summarized in table 2. The fact that in some of these experiments there was significant multiplication

TABLE 2

The growth-promoting effect of arginine on the Reiter treponeme

DATE	Heated rabbit serum, ml	Heated rabbit serum, dialyzed, ml	Heated human serum, ml	Thiogly-colate medium, ml	Sodium acetate (M/7), ml	Phosphate buffer (M/10.5), ml	Cysteine hydro-chloride (M/10.5), ml	Gluta-thione (M/7), ml	Ascorbic acid (M/7), ml	Initial number organisms (millions per ml)	0	0.1	0.2	0.5	1.0	1.5	2.0
				BASAL MEDIA						INITIAL NUMBER	FINAL NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION — ml of M/10 arginine						
2-28-44	1.0	0.5		0.3			0.33			4.3	0						116
8-11-44		1.0		0.3			0.33			5.4	35						110
3-20-44	0.6			0.3		0.5	0.33			5.4	1						47
8-30-44	0.4			0.3		0.5	0.33			5.0	14		63	75	84		
4-1-44	0.4			0.1		0.5	0.33			2.0	2						58
4-29-44	1.0			0.1	1.0	0.5	0.33			3.4	3			91			78
6-22-44	1.0			0.1	1.0	0.5	0.33			2.5	2	58					
	0.5	0.5		0.1	1.0	0.5	0.33			1.6	4						
				0.1	1.0	0.5				1.6	79			390			
				0.1	1.0	0.5				1.6	9			119			
11-24-45	0.1			0.05	1.0	0.5				3.0	14		93	107			
2-19-47	0.5			0.05	1.5	0.5		0.12	0.25	1.0	6					72	
6-24-47			0.5	0.1	1.5	0.5		0.12	0.25	1.4	3					132	

Arginine (M/10) was added as indicated on the right side of the table to the various basal media tabulated on the left side of the table. The experiments were similar to that recorded in table 1.

TABLE 3

Growth-promoting effect of arginine on the Reiter treponeme contrasted with that of a number of other amino acids and related compounds

COMPOUND	NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION
Agmatine	0
DL-α-Alanine	0
β-Alanine	1
DL-α-Aminobutyric acid	3
DL-β-Aminobutyric acid	8
ε-Aminocaproic acid	2
DL-α-Aminocaprylic acid	0
DL-α-Amino-α-ethylbutyric acid	6
α-Aminoisobutyric acid	7
DL-δ-Aminovaleric acid	1
L-Arginine	116
L-Arginic acid	9
DL-Aspartic acid	1
L-Benzoylarginamide	0
Betaine	0
DL-Citrulline	6
Creatine	3
Creatinine	8
L-Glutamic acid	4
Glycine	0
Glycocyamine	0
Guanidine	0
Guanidinovaleric acid	0
L-Histidine	3
Homoarginine	9
Homocysteine	6
Hydroxy-L-proline	0
Isoleucine	0
DL-Isovaline	3
L-Leucine	0
DL-Lysine	0
DL-Methionine	0
DL-Norleucine	1
DL-Norvaline	0
Ornithine	2
DL-Phenylalanine	0
L-Proline	8
Sarcosine	4
DL-Serine	0
DL-Threonine	0
DL-Tryptophane	2
L-Tyrosine	0
DL-Valine	3
Control	3

Various amino acids and closely related compounds in isotonic concentration and at a neutral pH were added in a volume of 2 ml to a basal medium consisting of 0.5 ml of heated rabbit serum, 0.3 ml of thioglycolate medium, 0.5 ml of $M/10.5$ phosphate buffer (pH 7.4), and 0.33 ml of $M/10.5$ cysteine hydrochloride. The inoculum yielded an initial count of 2 million organisms per ml in a total volume of 5 ml.

in the control tube containing no added arginine is perhaps to be attributed to the presence of sufficient amounts of arginine, or of an adequate substitute for it, in the particular lot of serum used in the medium, or in the growing culture used as an inoculum.

The unique activity of arginine is shown in table 3, in which are given the results obtained with 43 amino acids and related compounds. The initial inoculum throughout was 2 million organisms per ml; with arginine there were 116 million per ml after 6 days, but with the other substances tested the counts varied between 0 and 9 million per ml.

Acetic acid as a growth-promoting factor. When serum was dialyzed, it no longer permitted the growth of the Reiter organism in a thioglycolate-arginine

TABLE 4

The presence in whole serum of at least two distinct growth-promoting factors, one dialyzable and the other associated with the nondialyzable residue

DATE	BASAL MEDIA						INITIAL COUNT (MILLIONS/ML)	MILLIONS OF ORGANISMS/ML AFTER 6 DAYS' INCUBATION					
	Thioglycolate medium, ml	Arginine (M/10), ml	Phosphate buffer (M/10.5), ml	Cysteine hydrochloride (M/10.5), ml	Glutathione (M/7), ml	Ascorbic acid (M/7), ml		Serum fractions added to basal medium					
								Dialyzed serum	Serum ultrafiltrate	Serum dialyzate	Dialyzed serum + ultrafiltrate	Dialyzed serum + dialyzate	Whole heated rabbit serum
6- 5-44	0.15	0.5	0.5	0.33			3.3	2	2		145		140
2-19-44	0.20	1.0	0.5	0.33			1.0	10	1		97		114
4-26-44	0.05	1.0	0.5	0.33			0.5	12	1		71		70
6-19-47	0.20	1.5	0.5		0.12	0.25	2.0	30		3		245	310

Serum fractions obtained by ultrafiltration and by dialysis were tested separately and in combination, in conjunction with various basal media. Each serum component was added in an amount equivalent to 0.5 ml of whole serum, in a total culture volume of 5 ml. The original serum served as a control (last vertical column of table). The inocula were organisms sedimented from an actively growing culture on a serum-thioglycolate medium, and washed with basal medium before addition to the culture in order to remove the serum.

medium. The growth-promoting activity was, however, wholly restored by the addition of a serum ultrafiltrate or dialyzate (cf. table 4). Clearly, serum contributed at least two factors, one dialyzable and present in a serum ultrafiltrate, and one present in the residual nondialyzable fraction. Neither fraction alone supported the growth of the organism.

It was then found that the factor (or factors) present in the dialyzable or ultra-filtrable fraction of serum could be replaced by acetic acid. A typical experiment illustrating this point is summarized in table 5. In this experiment the addition of sodium acetate to dialyzed serum had a growth-promoting effect approaching that of serum dialyzate itself. As in the case of arginine, however, the acetic acid did not act as a trace factor. Although the smallest concentration exerting a significant effect was 0.0036 M, the amount of growth increased with the concentration of sodium acetate to reach a maximum at 0.06 M (0.5 per cent).

This is demonstrated by the data tabulated in table 6 and is graphically shown in figure 2. As in the case of arginine, the amounts of acetic acid necessary for maximum growth were out of all proportion to the amount of growth obtained. As in the case of arginine also, the growth-promoting activity of sodium acetate was quantitatively unaffected by recrystallization.

Unlike arginine, acetic acid was not unique in its growth-promoting activity.

TABLE 5

Acetic acid as a substitute for serum dialyzate in the cultivation of the Reiter treponeme

BASAL MEDIUM					INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS/ML AFTER 6 DAYS' INCUBATIO				
						Substances added to basal medium				
Thioglycolate medium, ml	Arginine (M/10), ml	Phosphate buffer (M/10.5), ml	Glutathione (M/7), ml	Ascorbic acid (M/7), ml		Sodium acetate (M/7), 1.5 ml	Dialyzed serum, 0.5 ml	Dialyzed serum, 0.5 ml + sodium acetate (M/7), 1.5 ml	Dialyzed serum, 0.5 ml + serum dialyzate 0.5 ml	Whole heated rabbit serum 0.5 ml
0.20	1.5	0.5	0.12	0.25	2.0	3	30	210	245	310

The serum components, sodium acetate (M/7), and inoculum were added to the basal medium under the same conditions as were used in the experiments summarized in table 4.

TABLE 6

The growth-promoting effect of acetic acid on the Reiter treponeme

DATE	BASAL MEDIA						INITIAL NUMBER OF ORGANISMS (MILLIONS PER ML)	FINAL NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION				
	Heated rabbit serum, ml	Heated rabbit serum dialyzed, ml	Thioglycolate medium, ml	Arginine (M/10), ml	Phosphate buffer (M/10.5), ml	Cysteine hydrochloride (M/10.5), ml		ml of M/7 sodium acetate (in total volume of 5 ml)				
								0	0.25	0.5	1	2
6-22-44	0.5		0.1	0.5	0.5	0.33	1.6	40			390	
7-18-44	0.5		0.1	0.2	0.5	0.33	6.0	15				114
6-14-44		1.0	0.1	0.5	0.5	0.33	4.4	0		4	29	
6-23-44		0.5	0.1	0.5	0.5	0.33	3.2	2	13		90	
6-30-44		0.5	0.1	0.5	0.5	0.33	1.4	0		12		34
—		0.5	0.1	1.0	0.5	0.33	0.5	0	12	21	36	55

Sodium acetate (M/7) was added as indicated on the right side of the table to the various basal media indicated on the left side of the table, all in a total volume of 5 ml. The inoculum was 0.1 ml of an actively growing serum: thioglycolate culture, representing an initial count of 0.5 million organisms per ml. The last horizontal row represents the experiment of figure 2.

Although its beneficial action was greater than that of any of the 10 homologous acids tested (formic, acetic, propionic, butyric, valeric, caproic, enanthylic, caprylic, pelargonic, and capric), a qualitatively similar effect was obtained with at least 15 of the 37 related compounds tested (cf. table 7). The results with many of these substances were obscured by their toxicity at the higher concentrations at which acetic acid was most effective.

Crystalline serum albumin as an adequate replacement for the nondialyzable frac-

tion of serum.   As previously indicated (table 5) the dialyzable fraction of serum essential for the growth of the Reiter spirochete could be replaced with acetic acid. It was subsequently found that the nondialyzable residue could be replaced with crystalline bovine serum albumin.   A typical experiment, in which a washed inoculum was used, is given in table 8.   A number of similar experiments that show the growth-promoting effect of serum albumin are summarized in table 9. The growth-promoting activity of serum albumin was usually not apparent until a concentration of about 0.1 per cent was reached, and increased up to the highest concentrations tested (1 per cent).

Sulfur-containing compounds as essential growth factors for the cultivation of the Reiter treponeme.   A series of experiments that demonstrate the essential role of cysteine are summarized in table 10.   In these experiments also, the organisms
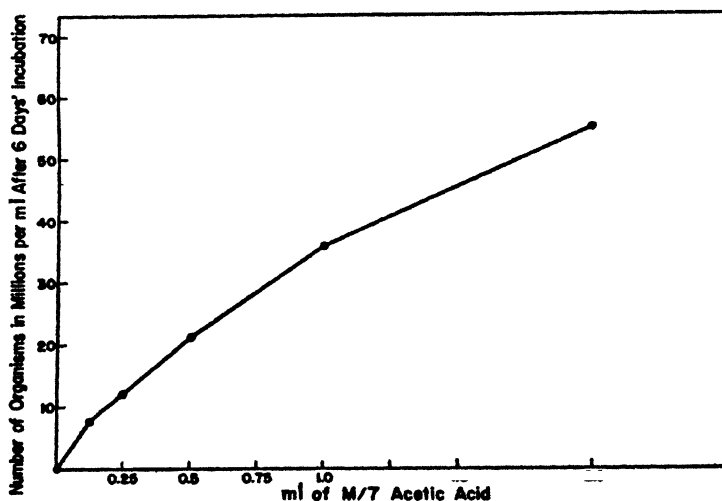
Figure 2. The growth-promoting effect of acetic acid on the Reiter treponeme as a function of its concentration.

were centrifuged and washed free of the culture medium in order to remove reducing substances.   That the function of cysteine was not merely the rapid induction of anaerobiosis in the culture medium was shown by the fact that when ascorbic acid was added to a concentration of 1:1,000 (sufficient to induce the rapid reduction of methylene blue or resazurin added as redox indicator in the culture media) no growth was obtained unless cysteine was also added.   Under such circumstances, as little as 0.0042 ml of an $M/10.5$ cysteine solution, equivalent to a final concentration of 0.00008 M, or 0.001 per cent, was able to promote growth in an otherwise —SH-deficient but anaerobic, culture medium.   Here also, the efficacy of the cysteine increased with increase in concentration up to the largest amount used (0.1 per cent).   A typical experiment illustrating this point is graphically shown in figure 3.

The data of table 11 (which includes the experiment of figure 3) show that

TABLE 7

The growth-promoting effect of acetic acid and related compounds on the Reiter treponeme

COMPOUND	NUMBER OF ORGANISMS (MILLIONS/ML) AFTER 5 DAYS' INCUBATION			
	ml of compound added			
	0.025	0.1	0.4	1.6
Acetic acid	7	8	12	36
Acetaldehyde	40	25	16	0
Acetamide	29	15	5	3
Acetone	15	28	—	31
α-Alanine	—	14	26	33
β-Alanine	17	—	16	20
Aspartic acid	7	—	—	6
Betaine	27	23	20	12
β-Bromopropionic acid	—	0	0	0
Chloral hydrate	12	13	0	0
Chloroacetic acid	8	0	0	0
Chloroacetamide	15	0	0	0
Choline	—	5	—	6
Creatine	10	8	—	9
Creatinine	10	6	—	4
Cyanoacetamide	38	15	—	13
Ethanolamine	—	0	0	0
Ethylamine	13	10	12	0
Ethyl acetate	24	30	21	0
Ethyl alcohol	16	16	18	21
Gluconic acid	6	6	—	5
Glucose	18	25	—	23
Glutamic acid	10	15	—	22
Glycerol	0	5	—	3
Glycine	8	9	3	0
Glycinamide	12	18	20	28
Glycocyamine	11	12	8	5
Glycolic acid	—	9	—	14
Lactic acid	—	15	17	20
Malonic acid	9	10	—	10
Oxalic acid	20	15	10	0
Phenylalanine	8	7	—	0
Pyruvic acid	21	—	23	40
Saccharic acid	6	—	3	3
Sorbitol	—	18	34	30
Succinic acid	—	8	9	15
Thioacetamide	16	18	22	10
Thioglycolic acid	20	5	3	0

A series of compounds in isotonic solution and at neutral pH were added to a basal medium consisting of 0.5 ml of dialysed heated rabbit serum, 0.1 ml of thioglycolate medium, 1.0 ml of M/10 arginine, 0.5 ml of M/10 phosphate buffer (pH 7.4), and 0.33 ml of M/10.5 cysteine hydrochloride. The initial count was 0.5 million organisms per ml in a total volume of 5 ml. The count after 5 days in a control tube containing no added compound was 3 million per ml.

TABLE 8

Serum albumin as an adequate replacement for the nondialyzable fraction of serum in the cultivation of the Reiter treponeme

BASAL MEDIUM						INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS/ML AFTER 6 DAYS' INCUBATION			
Thioglycolate medium, ml	Arginine (M/10), ml	Sodium acetate (M/7) ml	Phosphate buffer (M/10.5), ml	Gluta-thione (M/7), ml	Ascorbic acid (M/7), ml		Serum factors added to basal medium			
							Control (no added serum factors)	Crystalline bovine serum albumin	Dialyzed human serum	Whole heated human serum
0.20	1.5	1.5	0.5	0.12	0.25	2.0	3	148	208	310

In an experiment similar to that described in table 5, 0.5 ml of whole serum, 0.5 ml of dialyzed serum, and 0.5 ml of 5 per cent crystalline serum albumin, respectively, were added to the basal medium. The inoculum was a suspension (in the serum-free basal medium) of organisms sedimented from an actively growing culture and washed in the serum-free medium.

TABLE 9

The growth-promoting effect of serum albumin in conjunction with serum ultrafiltrate or sodium acetate in the cultivation of the Reiter treponeme

DATE	BASAL MEDIA							INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS PER ML AFTER 6 DAYS' INCUBATION					
	Thioglycolate medium, ml	Arginine (M/10), ml	Sodium acetate (M/7), ml	Phosphate buffer (M/10.5), ml	Cysteine (M/10.5), ml	Rabbit serum ultrafiltrate	Ox serum ultrafiltrate		ml of 5% crystalline bovine serum albumin (in total of 5 ml)					
									0	0.05	0.10	0.25	0.5	1.0
6-14-44	0.1	0.5	1.0	0.5	0 33			4.4	1					25
9-1-44	0.1	0.1	2.0	0.5	0.33	1.0		2.5	8					94
9-18-44	0.1	0.02	1.0	0.5	0.33	1.0		3.0	12					134
11-24-44	0.1	0.2	1.0	0.5	0.33			3.0	2			20	42	
2-19-45	0.2	1.0	2.0	0.5	0.33	0 5		0.1	0					34
2-19-45	0.2	1.0	2.0	0.5	0.33	0.5		1.0	0					89
4-9-45	0.05	1.0	1.0	0.5	0.33	0.5		0.5	0		9	19	25	35
4-16-45	0.05	1.0	1.0	0.5	0.33		0.5	0.5	0				26	
4-26-45	0.05	1.0	2.0	0.5	0.33		0.5	0.5	1	24	26		28	

Serum albumin (5 per cent) was added to the various basal media as described in the experiments of table 8.

TABLE 10

The essential growth-promoting effect of cysteine on the Reiter treponeme

DATE	BASAL MEDIA								INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS PER ML AFTER 6 DAYS' INCUBATION		
	Heated rabbit serum ml	Crystalline bovine serum albumin, ml	Rabbit serum ultrafiltrate	Thioglycolate medium, ml	Arginine (M/10), ml	Sodium acetate (M/10), ml	Phosphate buffer (M/10.5), ml	Ascorbic acid (M/7), ml		ml of M/10.5 cysteine		
										0	0.033	0.33
11-24-44	0.1			0.05	0.2	1.0	0.5		3.0	2		79
12-6-44	0.1			0.05	0.1	1.0	0.5		5.6	0		67
2-19-45		1.0	0.5	0.02	1.0	2.0	0.5	0.2	0.1	0	15	
2-19-45		1.0	0.5	0.02	1.0	2.0	0.5	0.2	1.0	0	37	

Cysteine (M/10.5) was added as indicated to the various basal media tabulated below, under the same general conditions as were used in the experiment summarized by table 1
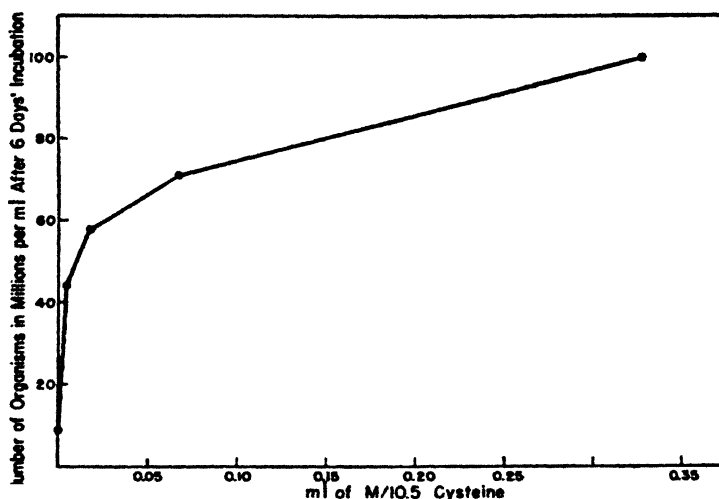
Figure 3. The growth-promoting effect of cysteine on the Reiter treponeme as a function of its concentration.

TABLE 11

The growth-promoting effect of various sulfur-containing substances

SULFUR-CONTAINING COMPOUND	MILLIONS OF ORGANISMS PER ML AFTER 6 DAYS' INCUBATION				REMARKS
	Final concentration of sulfur compounds				
	1:80,000	1:20,000	1:5,000	1:1,000	
Cysteine HCl	44	58	71	100	
Glutathione	32	45	67	71	
Homocysteine	32	—	—	37	Effective
Thioglycolic acid	40	48	41	45	
Thiamine	—	9	50	39	
Cystine	—	47	51	0	Effective,
Sodium sulfide	—	33	0	1	but toxic
Methionine	—	9	0	0	Ineffective

Various sulfur-containing substances at isotonic concentration and neutral pH were added as indicated to a basal medium consisting of 0.5 ml of 5 per cent crystalline bovine serum albumin, 0.05 ml of thioglycolate medium, 1.0 ml of M/10 arginine, 1.0 ml of M/7 sodium acetate, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and 0.2 ml of M/7 ascorbic acid, all in a total volume of 5 ml. The initial count was 0.5 million organisms per ml. The count after 6 days in a control tube containing no added sulfur-containing compound was 9 million per ml.

cysteine was not unique in its growth-promoting action. Not only were other sulfhydryl-containing compounds (glutathione, homocysteine, and thioglycolic acid) fully active, but several compounds in which the sulfur was present in an

—S— or —S—S— rather than —SH linkage (thiamine, cystine) were also effective. Methionine alone of the substances tested had no growth-promoting effect, perhaps because it is not transformed to an —SH compound under the conditions of the experiment.

Phosphate buffer. The phosphate buffer used in these experiments apparently had no effect over and above its buffering action. In several experiments the same culture medium was used with and without phosphate buffer, with no significant differences in the degree of multiplication. Also, the culture in the phosphate-free medium could be successfully subcultured into the same medium. These data indicate that the phosphate served only to maintain a pH range suitable for growth, and that the buffer otherwise had no beneficial action. This conclusion neglects the possibility that the minute amounts of phosphate probably present in the several unpurified components of the medium (yeast extract, trypticase) may suffice to exercise a growth-promoting effect. The point will require restudy when these as yet unidentified components can be replaced with chemically defined compounds.

DISCUSSION

It is clear from the data of table 3 that arginine alone of the amino acids here studied was essential for the growth of the Reiter strain of treponeme under the conditions of that experiment. The fact that such closely related substances as arginic acid, citrulline, and ornithine did not replace arginine suggests that arginine as such may enter the metabolic cycle of the organisms. However, the large amounts necessary in comparison with the amount of growth obtained suggest also that arginine may be merely substituting for another more active compound, as yet unidentified; and the very necessity for such large amounts may make it difficult to follow the metabolic pathways involved in its utilization.

Acetic acid was found to promote the growth of the Reiter organism under the experimental conditions. Although it was the only one of the low-molecular-weight saturated aliphatic acids tested which was active in this respect, acetic acid could be replaced by a number of closely related compounds such as acetaldehyde, ethanol, and pyruvic acid (see table 7). This may prove useful in tracing the metabolic pathways involved in the utilization of these compounds. Acetic acid (but not arginine) functioned as a substitute for the dialyzable components of serum; i.e., dialyzed serum or serum albumin gave good growth when supplemented with acetic acid on an otherwise inadequate basal medium (cf. tables 2, 5, 6, 7, 8, and 9).

The growth-promoting properties of both acetate and arginine were not manifest unless there were present adequate amounts of at least three other factors: (a) the unidentified factor (or factors) in the thioglycolate medium, (b) serum or serum albumin, and (c) cysteine or some other S-containing compound.

In agreement with previous reports (Little and SubbaRow, 1945; Whiteley and Frazier, 1948), crystalline bovine serum albumin (Armour) was found to be a

growth-promoting factor, and was shown to substitute for the nondialyzable fraction of whole serum (table 9). Its function is, however, not yet clear. Serum albumin plays an essential role in media developed for the cultivation of *Mycobacterium tuberculosis* (Dubos and Davis, 1946) and of *Trichomonas vaginalis* (Sprince and Kupferberg, 1947); and, in the case of the former medium, its favorable effect has been attributed primarily to a detoxifying action (Davis and Dubos, 1947). Studies are now in progress to determine its function in the cultivation of the Reiter organism.

Five components have therefore been identified as essential and together to be adequate for the multiplication of the Reiter treponeme in an anaerobic environment: (1) arginine, (2) acetic acid, (3) any one of a number of sulfur-containing substances, (4) crystalline serum albumin, and (5) an as yet unidentified component (or components) in the thioglycolate medium. Of the four chemically defined compounds only the —SH—containing substances were effective in such small concentrations (0.00008 M) as to suggest that they might act as growth factors rather than metabolites. The present experiments furnish no evidence as to whether the other substances serve merely as sources of energy or are used in building up cell protoplasm.

The "thioglycolate medium" which must still be added apparently contains only three substances that could be contributing to growth: a yeast extract, an enzymatic casein digest (trypticase), and glucose. (The phosphate, resazurin, and agar in the thioglycolate medium have been shown to be probably noncontributory under the conditions of the present experiments; and the cystine and thioglycolic acid have been shown to be replaceable by any sulfhydryl-yielding compound.) Since the yeast extract and casein digest permit growth of the organism when they are added to the other four ingredients in final concentrations of 1:10,000 and 1:3,300, respectively, the active component (or components) must be effective in minute concentration. Studies on the identification of those active components are now in progress.

SUMMARY

A mixture of acetic acid, arginine, any one of a number of sulfur-containing compounds, and crystalline serum albumin, supplemented with minute amounts of yeast extract, an enzymatic casein digest, and glucose, has been shown to permit the multiplication of the Reiter treponeme.

The acetic acid has been shown to replace the dialyzable components of whole serum, and crystalline albumin has been shown to substitute for the nondialyzable fraction.

The minimal effective concentrations of acetic acid and arginine were 0.0036 M and 0.0005 M, respectively, but the rate of growth increased progressively with increasing concentrations up to the highest concentrations studied. The minimal effective concentrations of the sulfhydryl compounds studied were of the order of 0.0001 M, and serum albumin was effective at a concentration greater than 0.1 per cent.

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A QUANTITATIVE ANALYSIS OF THE RESISTANCE OF MYCOBACTERIA TO STREPTOMYCIN[1]

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The rapid rate at which bacteria develop resistance to streptomycin has been demonstrated *in vitro* and *in vivo*. Quantitative studies have been made of certain species of non-acid-fast organisms which give rise to streptomycin-resistant variants (Klein, 1947; Alexander and Leidy, 1947; Klein and Kimmelman, 1946). These investigations show that small numbers of resistant cells are present in susceptible populations *independent* of the presence of the drug, and it is now generally agreed that these arise by a process of spontaneous mutation. Naturally resistant forms of tubercle bacilli have been isolated by Pyle (1947) directly from the sputa of patients who have not been treated with streptomycin, and the presence of naturally resistant variants in stock cultures of H37RV has been demonstrated by Vennesland, Ebert, and Bloch (1947).

This is a report of studies made of the orderly manner in which spontaneous variants occur in cultures of *Mycobacterium tuberculosis* and *Mycobacterium ranae*. The quantitative data were obtained from the many tests required to establish figures large enough to bear statistical analysis. These data seem to provide a reasonable explanation for the variation in resistance encountered in the cultures of patients who have been treated with identical therapeutic regimens. In addition, it will be shown that the number of variants in a given susceptible population is directly affected by the concentration of the drug present in the medium. Similarly, the incidence of variants is increased as the size of the population increases, provided the concentration of the drug remains constant. These and other findings will be discussed in this report.

The quantitative study was made by growing the microorganisms in liquid tween albumin medium (Dubos and Davis, 1946). Following this, known numbers of cells were plated on solid medium containing specific concentrations of streptomycin. The progeny of organisms growing in the presence of the drug were further analyzed to determine their resistance to various concentrations of streptomycin. The details of the experimental procedures are given under Materials and Methods.

MATERIALS AND METHODS

Tubercle bacilli used in this study were strain H37RV and two strains, WS and WR,[2] isolated from patients' sputa. The strains were obtained from patients with advanced pulmonary tuberculosis prior to the institution of treatment with streptomycin. Although both patients received ⅓ gram of streptomycin every

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4 hours for 120 days, organisms from the sputa of WS were resistant to 1,000 micrograms per ml of streptomycin after 30 days of treatment, whereas organisms from the sputa of WR remained sensitive to 1.0 to 2.5 micrograms per ml of streptomycin after the 120 days of treatment were completed. In addition, *M. ranae* was used. Each of the strains was grown on Hohn's medium, and these cultures served as the source of organisms for subsequent experiments.

The media used in this investigation are liquid tween albumin and a solid tween albumin medium prepared as follows:

   I. Concentrated mineral base:

      KH_2PO_4 .... 1 g
      Na_2HPO_4 $12H_2O$ .... 6.25 g
      Na-citrate .... 1.5 g
      Ferric ammonium citrate .... 0.1 g

   Dissolve in 50 ml of distilled water.

   Dissolve 0.6 grams $MgSO_4 \cdot 7H_2O$ in 50 ml distilled H_2O. Mix the two solutions.

  II. To 100 ml of concentrated mineral base add 900 ml H_2O.

   Add asparagine .... 4 g
      "Tween 80" (10 per cent in H_2O) .... 5 ml

   pH adjusted to 7.0
      Add agar .... 15 g

   Autoclave, cool to 60 C, and add 100 ml of bovine albumin (5 per cent in 1.5 per cent NaCl, Seitz filtered and heated at 55 C for 30 minutes). Add sterile 25 per cent glucose solution, 20 ml. Add streptomycin as needed.

   The preparation is shaken well, and after streptomycin is added plates are poured containing about 25 ml each of the medium.

A flask of liquid medium inoculated from a stock culture is incubated, with occasional shaking, for from 7 to 10 days until a growth of the desired turbidity is obtained. The flask is then shaken well for at least one-half hour to obtain an even suspension of cells. Following this, the number of cells per ml is determined by diluting by the usual methods and then plating on the surface of the solid medium. One ml of a liquid culture to which streptomycin has been added until the desired concentration of the drug is obtained is placed on a series of plates containing the same concentration of streptomycin. After being tilted to ensure uniform spread of the inoculum, the plates are placed in the incubator until the excess moisture has evaporated and then are sealed with wide rubber bands. The final reading of colonies growing on plates is made after approximately 35 days' incubation.

To analyze further the resistance of a colony growing in a known concentration of streptomycin, the colony is inoculated into liquid medium containing no streptomycin. When the desired growth is obtained, the cells are plated on solid medium and on plates containing the same and higher concentrations of streptomycin. The plates are incubated as in the procedure mentioned above and the results tabulated.

The general procedure when *M. ranae* is employed is the same as the foregoing procedure outlined for *M. tuberculosis* with two exceptions: plain glycerol agar is used in place of solid tween albumin medium; and the incubation period before counting the colonies is from 5 to 7 days.

TABLE 1

Mycobacterium tuberculosis H37RV

DISTRIBUTION OF COLONIES AMONG PLATES

Number of bacteria per inoculum	Number of colonies growing on each plate containing 1 microgram of streptomycin per ml of medium										Total number of resistant colonies
208 billion	10	15	6	16	11	13	15	18	10	19	
	17	8	5	12	14	14	17	19	9	10	
	11	12	14	15	5	3	13	12	15	17	375
	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
298 billion	2	1	1	0	0	1	2	4	1	2	
	3	4	2	0	1	2	1	1	1	2	
	1	1	1	2	0	3	3	2	4	2	
	1	0									51
149 billion	1	0	0	0	2	2	0	1	1	0	
	1	0	1	0	0	1	0	1	1	2	
	0										14
10 billion	0	0	0	0	0	0	0	0	0	0	0
	Number of colonies growing on each plate containing 1,000 micrograms of streptomycin per ml of medium										
208 billion	1	1	0	0	2	0	2	2	0	0	
	1	1	1	0	0	2	0	1	0	1	
	1	2	1	0	2	0	1	1	0	1	24

RESULTS

The results of the experiments are arranged in the four tables. In table 1 showing the results obtained with H37RV, it is to be noted that as the size of the sample of organisms increases, the number of variants resistant to 100 micrograms per ml of streptomycin is significantly increased. With samples of the same size there is great variation in the number of resistant colonies appearing on any one plate. Several plates contain no colonies. As the size of the sample is reduced, the number of plates showing no growth is increased; and when the sample size is reduced still further, the chances of growth on a plate become negligible. When the concentration of the drug in the medium is reduced to 1 microgram per ml, the number of resistant colonies growing is increased and every plate contains some colonies; but when the concentration of the drug is increased

to 1,000 micrograms per ml, there is no significant change in the number of naturally resistant colonies from the number growing in 100 micrograms per ml. The samples listed in table 1 were obtained from the same liquid culture. When other cultures of the same strain are used at different times, the figures obtained will vary but the fundamental relations between sample size and concentration of drug will hold.

TABLE 2

Mycobacterium tuberculosis strains WR and WS

Number of bacteria per inoculum	DISTRIBUTION OF COLONIES AMONG PLATES										Total number of resistant colonies
	Number of colonies growing on each plate containing 1 microgram of streptomycin per ml of medium										
Strain WR											
499 billion	0	1	3	2	0	2	1	2	1	1	
	3	2	1	4	2	0	2	3	2	1	54
	4	3	1	2	0	3	3	2	2	1	
	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
998 billion	0	2	1	1	0	0	0	0	0	0	
	3	0	1	0	0	1	1	1	3	0	19
	1	0	0	1	0	0	2	0	1	0	
Strain WS											
	Number of colonies growing on each plate containing 1 microgram of streptomycin per ml of medium										
200 billion	4	5	3	2	5	1	4	6	0	1	
	1	5	3	1	6	4	5	4	8	2	70
178 billion	4	3	12	12	15	10	7	5	4	13	
	5	4	3	2	16	15	1	20	18	10	179
	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
400 billion	2	1	1	2	3	2	3	3	0	1	
	2	0	0	2	1	1	6	2	3	0	35
178 billion	1	2	2	1	0	0	3	1	2	4	
	3	2	4	4	3	0	3	6	5	1	47

A comparison of the results shown in table 2, obtained with strains WS and WR, confirm the prediction that more resistant variants would be found in strain WS, the strain which developed resistance to streptomycin after 30 days of treatment of the patient. Because of population changes due to subculturing, these results may not always be predicted (see Discussion). When samples from different subcultures of strain WS were used, the number of resistant forms, as noted in table 2, varied greatly in number. Although the results in the table show the

TABLE 3

Mycobacterium ranae

DISTRIBUTION OF COLONIES AMONG PLATES

Number of bacteria per inoculum	Number of colonies growing on each plate containing 1 microgram of streptomycin per ml of medium										Total number of resistant colonies
12 billion	1	0	2	1	0	0	0	1	2	0	
	0	1	1	0	0	1	0	2	0	1	
	1	1	2	0	1	1					19
210 billion	5	13	8	5	5	14	7	3	6	11	
	10	10	8	6	9	10	7	11	9	10	
	15	7	8	12	5	7					221

	Number of colonies growing on each plate containing 10 micrograms of streptomycin per ml of medium										
88 billion	1	5	0	1	1	0	0	1	3	1	
	1	0	1	2	2	2	1	0	2	4	
	1	0	5	1	1	2					38
120 billion	5	3	2	0	1	0	3	2	1	0	
	0	4	0	3	3	0	1	1	3	4	
	1	0	5	1	1	2					46
42 billion	0	0	1	2	0	1	0	0	1	2	
	0	0	0	0	1	1	0	2	0	0	
	1	1	0	0	0	0					13

	Number of colonies growing on each plate containing 100 micrograms of streptomycin per ml of medium										
14 billion	0	0	0	0	0	0	0	0	0	0	
	1	0	0	0	0	0	0	0	0	0	
	0	0	0	0	0	0	0				1
168 billion	0	0	1	0	2	0	0	0	1	0	
	1	1	0	0	0	1	1	0	0	0	
	0	0	1	0	0	1	0	0			10
115 billion	2	0	0	1	2	0	0	0	0	1	
	1	0	0	0	0	0	1	2	0	0	
	0	0	1	0	2	0	0	0			13
453 billion	1	2	4	0	1	3	0	0	4	3	
	2	4	1	0	3	1	1	0	0	0	
	1	2	0	0	1	2	0	0			36

number of resistant variants to be increased when strain WS was subcultured, other samples of subcultures of the same strain have been shown to contain fewer variants.

In table 3 are recorded the results when *M. ranae* was used; and it can be readily

TABLE 4

Analysis of the resistance to streptomycin of colonies growing in the presence of 1 unit of streptomycin per ml of medium

CONCENTRATION OF STREPTOMYCIN IN MICROGRAMS PER ML OF MEDIUM	NUMBER OF BACTERIA
I. *M. tuberculosis* strain H37RV	
A. 0	210 billion
1	226 billion
10	198 billion
100	2,000
1,000	200 (to 2,000)
B. 0	1.2 million
1	1.4 million
10	980,000
100	0
1,000	0
II. *M. tuberculosis* strain WR	
0	78 billion
1	81 billion
10	67 billion
100	305
1,000˙	125
III. *M. tuberculosis* strain WS	
0	128 billion
1	109 billion
10	101 billion
100	684
1,000	278
IV. *M. ranae*	
0	1.9 billion
1	1.6 billion
10	1.8 billion
100	400
1,000	10

Analysis of the resistance to streptomycin of colonies growing in the presence of 100 units of streptomycin per ml of medium

I. *M. tuberculosis* strain H37RV	
A. 0	26 million
100	26 million
1,000	25 million
B. 0	228 billion
100	225 billion
1,000	210 billion
II. *M. ranae*	
0	31 million
100	30 million
1,000	32 million

seen that the general relationships between the number of organisms in a sample concentration of drug and the number of resistant colonies hold for this organism. The data obtained with *M. ranae* are probably more accurate than those with *M. tuberculosis* because the size of the sample is more accurately determined for *M. ranae*. With growth of this organism in liquid media cells show no clumping, whereas with *M. tuberculosis* microscopic clumping is occasionally seen even after thorough shaking.

In analysis of the resistance of colonies growing on plates containing 1 microgram per ml of streptomycin, table 4 shows that all the organisms are resistant to 1 microgram per ml of streptomycin. It is of interest that the number of organisms resistant to higher concentrations of streptomycin is greater among these organisms resistant to 1 microgram per ml of streptomycin than it is among the original parent population. (See tables 1, 2, 3 for comparison.)

DISCUSSION AND SUMMARY

From the foregoing observations it seems evident that the nature of resistance of mycobacteria to streptomycin is that of selection of normally occurring hereditary variants that are present in the original cell population. That the addition of streptomycin to the medium renders it selective for the growth of resistant variants cannot be disputed. The specific induction of such forms by the drug can be seriously questioned since the number of variants present in an original population is so small. Furthermore, it was shown previously by Yegian, Budd, and Middlebrook (1946) that tubercle bacilli exposed to sulfonamides under conditions precluding multiplication fail to develop resistance. Additional studies using quantitative methods with streptomycin showed that no increase in the number of resistant variants occurred under these conditions. The facts that the resistant variants are genetically stable and that they are present in small numbers in a random population support the contention that they are produced by a process of mutation regardless of the presence of the drug.

It is evident from an examination of the data that an increase in the population of one parent strain is accompanied by an increase in the number of variants resistant to a specific concentration of the drug. It is to be noted, however, that the populations in different cultures show a great variation in the number of resistant variants present. Even the samples taken from the same culture show a great variation in the number of resistant forms. These findings are readily explained on the basis of chance selection.

The data presented show clearly that the number of resistant forms is directly affected by the concentration of the drug in the media. A marked decrease in the number of colonies occurs when the drug concentrations are increased from 1 microgram per ml to 10 micrograms per ml and then to 100 micrograms per ml; however, when certain high concentrations of the drug are used, such as 100 micrograms per ml and 1,000 micrograms per ml, no significant difference in the number of resistant colonies is observed.

From analyses of populations resistant to 1 microgram per ml of streptomycin, it is evident, as expected, that all the organisms are resistant to 1 microgram per

ml of streptomycin, and that there are more highly resistant variants in the populations resistant to 1 microgram per ml than in the original parent populations. The elimination of all organisms susceptible to 1 microgram per ml of drug selects a population all of whose members are resistant to streptomycin to some degree; this would appear to facilitate not only the multiplication of the (relatively few) highly resistant forms already present but possibly the production by mutation of more highly resistant forms. From his studies of resistance of bacteria to penicillin Demerec (1945) concluded that the degree of resistance to the drug increases by definite increments as the naturally resistant forms multiply. A similar process may be occurring during our analyses of resistant populations. Also, it is observed from the data presented that variants resistant to 100 micrograms per ml of streptomycin are equally resistant to much higher concentrations of the drug. Apparently streptomycin in any concentration has no effect on forms resistant to a certain minimum concentration of the drug.

The clinical significance of the findings presented must be emphasized, for often data concerning the sensitivity of tubercle bacilli to streptomycin are either inadequate or improperly interpreted. It has been shown that, when the sample of bacteria used is sufficiently large and when the time allowed for growth is prolonged, the chances of finding resistant forms are increased. As the sample size decreases, the chances of resistant forms being observed are greatly decreased. When only one sample is used, the conclusions drawn must be very limited, for we have noted great variations among samples from the same parent population. When sensitivity studies are made in the usual manner, a time limit is used for allowing growth of resistant forms. When the time limit is extended, a few resistant forms may be allowed to multiply adequately and the results may be given a quite different interpretation. In addition, the collection of sputa specimens from which the original stock cultures are grown must be considered inadequate in certain instances. All the variations in resistance of the bacterial population in the diseased areas of the patient are probably never represented in any single or even in several sputa samples.

It is known that multiplication of cells is essential to increase the number of resistant forms. The reproduction rate of tubercle bacilli within patients under treatment with streptomycin may vary greatly in different patients; in addition, the reproduction rate of the stock cultures and subcultures varies greatly. Of great importance is the character of the individual population. The mutation of resistant forms is very dependent upon this factor, which is difficult to analyze.

When tubercle bacilli strains WS and WR were studied prior to streptomycin treatment of the patient (table 2), the predication was verified that more resistant variants would be found in strain WS, the strain which later developed resistance to streptomycin after 30 days of treatment of the patient. Numerous subcultures of the strains were made, and because of population changes the results might have been different. Distinct variations in the number of resistant forms found in strain WS were noted when different subcultures of the same strain were used. Some samples showed definite decreases in the number of variants present.

It is conceivable that all the bacteria within a patient are not exposed to the same concentration of streptomycin. Because of a low concentration of streptomycin in certain of the diseased areas, bacteria which may be readily susceptible to streptomycin are allowed to exist during the treatment period. The presence of these organisms in the sputum at a later date may go undetected during routine sensitivity studies because the resistant forms outnumber these remaining sensitive organisms and because the procedure is designed to "select out" only the resistant forms. It is conceivable, also, that an extension of disease in the patient after the treatment period is concluded could be caused by these streptomycin-susceptible organisms. It is essential that complete studies be made of tubercle obtained from many different areas in patients who have undergone treatment with streptomycin and subsequently died.

It is apparent that many factors can influence the results obtained in the usual sensitivity studies. It must be borne in mind constantly that chance and chance alone may account for the findings obtained. Sample variations, the multiplication of the cells, the time allowed for growth, and the characteristics of an individual population must always be considered.

The demonstration of the resistant variants in a random population, as has been shown, is a relatively simple procedure. It is known that resistance is specific and permanent in nature. Is it not possible that the resistant variants may occasionally give rise to mutants resembling the parent strain in their susceptibility to streptomycin? Study of this problem is rendered difficult because there is no selective medium that prevents growth of the resistant strain and allows growth of the streptomycin-susceptible strain that may have mutated from the resistant strain. The problem resolves itself into isolating from a resistant strain still another variant that requires streptomycin for its growth. Isolation of such a variant of *M. ranae* has been accomplished. By isolating this variant we have obtained an organism for which a selective medium exists (Yegian and Budd, 1948). From studies of large populations of this variant strain that requires streptomycin for its growth, a few colonies have been isolated that resemble the parent strain in their susceptibility to streptomycin and sulfonamides, in their biochemical reactions, and in their morphological and cultural characteristics. These findings are similar to those obtained by Miller and Bohnhoff (1947) in their studies of meningococcus.

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THE ENDOTOXIN OF PASTEURELLA PSEUDOTUBERCULOSIS

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Natural infections with *Pasteurella pseudotuberculosis* have been recognized in man and in numerous species of lower animals since the original description of the organism by Malassez and Vignal in 1883. Although Dessy (1925) and Meyer (1928) have mentioned the presence of toxic substances in broth cultures of *P. pseudotuberculosis*, the properties of such substances have apparently not been recorded in the literature.

MATERIALS AND METHODS

Cultures. The 27 strains of *P. pseudotuberculosis* previously studied by Lazarus and Gunnison (1947) were investigated for ability to produce endotoxin, using the methods described below. Two strains, "Saranac" and "New Orleans," were found to produce endotoxin. The reasons for 2 strains being toxigenic and the remaining 25 lacking this property have not as yet been adequately investigated.

Strain Saranac was received in 1944 from Dr. Leroy U. Gardner of the Trudeau Foundation of Saranac Lake, New York. The organism was isolated from cases of pseudotuberculosis occurring in the animal colony of the Saranac Laboratory. The culture has been carried on various media since 1944, most frequently on Difco proteose no. 3 agar. The organism apparently belongs to serologic group I, as classified by the system suggested by Schütze (1928).

Strain New Orleans was isolated from a fatal human case and described in detail by Moss and Battle (1941). The organism probably belongs in serologic group III.

Bacteriophage. The strain of phage used in this study was originally isolated from a clinical case of bubonic plague by Advier (1933). The adaptation of the phage to *P. pseudotuberculosis* and its general properties have been previously described (Lazarus and Gunnison, 1947).

Production of endotoxin. Difco proteose no. 3 broth was the standard medium used. Broth cultures were prepared by inoculating heavily with a young culture of the desired strain and incubating 8 hours at 37 C. To the turbid culture was then added undiluted phage in a volume equal to 2 per cent of the volume of the culture. Additional overnight incubation resulted in a decrease in turbidity, the final appearance of the medium varying with different strains from partial to complete clarity. The lysates were passed through a Seitz EK filter disk and stored in the refrigerator, at 0 C or at +10 C.

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RESULTS

The following observations apply to the endotoxin of both Saranac and New Orleans strains.   No basic differences in effect or in potency have been observed.

Properties of the endotoxin.   Lysates produced as described above were fatal to mice in from 2 to 48 hours following intravenous inoculation of 0.5 ml.   Although there was variation in toxicity with different lots, dilutions of 1 in 6 to 1 in 8 usually killed 50 per cent of the mice used.   For purposes of this study, deaths 48 hours or more after inoculation were not considered as being due to endotoxin.

The method of preparing endotoxin by allowing broth cultures to autolyze at 37 C was not successful with *P. pseudotuberculosis*.   Although filtrates of autolyzed cultures definitely yielded endotoxin, the amount when titrated in mice was no greater after 5 to 10 days in the incubator than was produced by overnight lysis of an 8-hour culture through the agency of phage.   Daily filtrates of cultures held at 37 C showed a progressive decrease in endotoxin content after 10 days, until in 15 days the filtrate no longer was able to kill mice.   This was interpreted as evidence of the instability of the endotoxin, which was further demonstrated by its relatively rapid destruction by heat or prolonged storage.

Lysates originally capable of killing mice within 48 hours following intravenous inoculation were inactivated after exposure to 60 C for 30 minutes, and no longer produced any effect in animals.   Storage at +10 C resulted in inactivation of the endotoxin in approximately 8 months without any marked effect on the phage contained in the same preparation.

The apparent lability of the endotoxin of *P. pseudotuberculosis* is at variance with the heat stability of most other endotoxins, although other exceptions have been reported (Topley and Wilson, 1946).   The endotoxin described in this report differs somewhat in heat sensitivity from the endotoxin of *Pasteurella pestis*, which is not completely inactivated after 4 hours at 56 C (Meyer, 1948).

Animal inoculation.   Rabbits injected intravenously with a single 5-ml dose of endotoxin died in 6 to 8 hours.   Autopsy showed circulatory changes, particularly in the abdominal region, that have been recognized as typical of endotoxin death (Delaunay, Lebrun, and Cotereau, 1947).   The blood vessels of the abdominal wall, omentum, and intestines were engorged, and areas of hemorrhage were macroscopically visible.   Fluid tinged with blood was present in the abdominal cavity, and to a lesser degree in the thoracic cavity.   The smaller blood vessels of the thoracic area showed some hemorrhages macroscopically.   Death was apparently due to major circulatory changes, which Delaunay *et al.* have suggested are similar to those seen in traumatic shock.

In guinea pigs and mice, the general picture was similar to that in the rabbit. No characteristic changes were visible in the organs, the circulatory system being the major site of difficulty.   Although intravenous inoculation of endotoxin produced the most rapid death, intraperitoneal or subcutaneous injections were likewise fatal, with the same general signs of circulatory damage.

Inoculation of from 0.05 to 0.1 ml of endotoxin intradermally into shaved guinea pigs produced an erythematous reaction reaching a maximum diameter of

2 to 3 cm in 48 hours and progressing to central necrosis, formation of a scab, and subsequently a smooth scar. There was no evidence of local resistance to the endotoxin, as demonstrated by second inoculations of an area previously used for a positive intradermal reaction. It is of importance to note that the two cultures producing the lethal factor were the only two to produce any reaction in the guinea pig skin, the lethal and necrotizing factors probably being merely different manifestations of the endotoxin.

Attempts to produce antitoxin. The endotoxin was readily detoxified by the addition of 0.3 per cent formalin. After 48 hours at 37 C, the treated endotoxin was harmless for mice when inoculated intravenously in 0.5-ml amounts.

No evidence of antitoxin production in rabbits could be demonstrated following 7 injections of such detoxified endotoxin spaced over a period of 3 weeks. The serum from these rabbits not only failed to neutralize small amounts of endotoxin, as shown by intravenous inoculation of the mixture into mice, but in addition the rabbits succumbed to small doses of toxin. One-half ml of endotoxin intraperitoneally was fatal to these rabbits, with typical autopsy findings as described above. Similar findings were noted in mice, with the evidence indicating that 1 or 2 doses of detoxified material were unable to stimulate antibody production sufficient even to neutralize a single lethal dose of toxin. The possibility that better results could be obtained following more numerous injections of endotoxin treated with formalin is being investigated further.

<div align="center">DISCUSSION</div>

The lethal properties of phage lysates from two strains of *P. pseudotuberculosis* are not connected with the phage, but rather with the bacterial cells and the products of their lysis. This is demonstrated by the fact that phage lysates of 25 additional cultures of the same organism were unable to produce either the lethal or necrotizing factors, although both phage and lysed bacteria were present in the same amounts as in the two toxigenic cultures. Furthermore, cultures of the two toxigenic strains produced endotoxin when autolysis was accomplished without phage. It seems likely that the amount of endotoxin obtained from the two toxigenic strains could be increased by using methods other than phage lysis or autolysis as a result of age.

The endotoxin of *P. pseudotuberculosis* produces in animals the typical effect on the circulatory system which has been reported for other endotoxins. It likewise shows the inability to give rise to specific symptoms or lesions that is a general attribute of the group of endotoxins (Topley and Wilson, 1946). The heat sensitivity of the endotoxin of *P. pseudotuberculosis* is somewhat greater than that of the endotoxin of *P. pestis*, and may offer additional evidence that the two organisms are not identical.

It is of interest to note that Moss and Battle (1941), in reporting on the isolation and identification of the New Orleans strain, mention death of guinea pigs in 12 hours following subcutaneous or intraperitoneal inoculation of whole organisms. This was undoubtedly the result of endotoxin action, in the light of the foregoing data.

SUMMARY

An endotoxin was prepared by lysis of two different strains of *Pasteurella pseudotuberculosis* with bacteriophage. The endotoxin produced major circulatory changes and death in less than 12 hours in rabbits, guinea pigs, and white mice. In addition to death of experimental animals, the endotoxin in small doses was able to produce necrosis in the skin of guinea pigs.

The endotoxin was inactivated by heating for 30 minutes at 60 C, being slightly more sensitive in this respect than the endotoxin of *Pasteurella pestis*.

Endotoxin, after detoxification with formalin, was ineffective in producing antitoxin, no evidence of protection being obtained.

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THE PARTIAL PURIFICATION OF STAPHYLOCOAGULASE AND THE EFFECT OF CERTAIN PRESUMPTIVE INHIBITORS UPON ITS PLASMA-COAGULATING ACTION[1]

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Staphylocoagulase (Gratia, 1919) appears to require a cofactor present in plasma (Smith and Hale, 1944). Gerheim, Ferguson, and Travis (1948) propose "prostaphylocoagulase" as the name of the staphylococcal product, which would tacitly leave staphylocoagulase as the term indicating the active adduct of bacterial product and plasma cofactor. The plasma cofactor has been reported to be associated with plasma albumins (Gerheim, Ferguson, and Travis, 1947) and with plasma globulins (Tager, 1948).

The coagulation of plasma by living cultures of staphylococci is not inhibited by citrate, hirudin, fluoride (Much, 1908), oxalate (von Gonzenbach and Uemura, 1916), heparin (Rigdon, 1942), cobra venom, chlorazol fast pink, hydroquinone, or low oxygen tension (Walston, 1935). Gengou (1933) reported no inhibition following storage of broth cultures of staphylococci in the presence of 0.15 per cent phenol, 0.1 per cent permanganate, or 0.1 per cent trypaflavine, but complete inactivation by 0.3 per cent formaldehyde in 48 hours at 37 C. Neter (1942) reported that zephiran chloride 1:10,000 prevented, and 1:100,000 delayed, coagulation of oxalated human plasma by a broth culture of living staphylococci. Adequate controls ruled out any effect of zephiran at these concentrations on the coagulability of the plasma itself; the experiments were not decisive as to whether the action was on the organism or on the staphylocoagulase. Spink and Vivino (1942) found that sulfathiazole (in concentrations of 100 to 1,000 mg per 100 ml) accelerated the clotting action of sterile broth filtrates of coagulase-positive strains of *Staphylococcus aureus* on citrated human plasma. A similar effect of slightly less magnitude was shown by sulfanilamide; on the other hand, sulfapyridine was slightly, and sulfadiazine strongly, inhibitory to coagulation. All the sulfonamides studied showed an accelerating action in the presence of *p*-aminobenzoic acid, which by itself also had an accelerating action. Lominski and Roberts (1946) found, in 212 out of 348 human sera, a substance that neutralized coagulase. The inhibitory substance is precipitated with the serum globulins by ammonium sulfate and resists heating at 63 C for 30 minutes. Agnew, Kaplan, and Spink (1947) tested coagulating action and viability of staphylococci in the presence of penicillin and streptomycin. Penicillin inhibited growth in concentrations of 3,125 units (1.87 mg crystalline penicillin G sodium) per ml or higher; the inhibition of coagulase was minimal at a concen-

tration of 49 units per ml, and almost complete at 25,000 units (15 mg) per ml. Streptomycin inhibited growth in concentrations of 4 mg per ml and higher; coagulation was inhibited in all concentrations studied, the lowest being 0.25 mg per ml. Farkas (1947), using Berkefeld filtrates of *S. aureus* cultures, found that bromine in a concentration of 0.3 per cent or more would inhibit plasma coagulation, and that similar inhibition occurred with 0.8 per cent iodine. The same author reports an inhibitory effect with tetrathionate, but does not specify the amounts used.

PREPARATION OF CONCENTRATED STAPHYLOCOAGULASE

The procedure which follows yields a concentrate of the bacterial product commonly called staphylocoagulase, possibly better called prostaphylocoagulase. A strongly coagulase-positive strain of *S. aureus* (which strain we designate arbitrarily as "L" and which was originally isolated from a chronically infected mastoid wound) is grown for 1 week in 100-ml portions of beef heart tryptic digest medium (pH 7.6). The cultures are then autoclaved 20 minutes at 120 C. This drastic step is justified by the observation of Gengou (1933) that the coagulating substance is highly resistant to thermal inactivation; it is easily inactivated by proteolytic enzymes (Walker, Schaffer, and Derow, 1947). Autoclaving has the advantages of removing certain interfering proteins by coagulation, rendering the cocci more readily separable in the centrifuge, and removing the hazard of contaminating the subsequent preparations with living cocci. The supernatant of the autoclaved cultures is adjusted to pH 4 with hydrochloric acid at room temperature, cooled to zero C, and centrifuged at that temperature. The precipitate is washed three times with one-tenth the original volume of filtrate: first with cold sodium acetate buffer, pH 4, ionic strength 0.1 (Boyd, 1945); next with cold acetate buffer of the same pH, ionic strength 0.01; and finally with cold distilled water. It is then suspended in a convenient volume (about 50 ml) of distilled water at room temperature and brought to pH 7.5 with sodium hydroxide, spun at room temperature, and the small insoluble residue discarded. The supernatant is adjusted to pH 4, centrifuged, and washed as before. It is finally resuspended in a minimal volume of distilled water, redissolved at pH 7.5, and dried in a vacuum from the frozen state. The product is promptly water soluble; it is not consistently stable either when dry or in solution. Depending upon the potency of the original culture supernatant, final yields vary between 25 and 60 mg per 100 ml of original supernatant, containing from 4 to 10 mg of nitrogen.

Coagulase activity of solutions of this product is determined by the same serial dilution procedure previously described (Walker, Schaffer, and Derow, 1947). The extent of purification is indicated by the determination of the ratio $\frac{\text{coagulase titer}}{\text{mg of nitrogen}}$ which in crude supernatants has a value of about seven and in our best preparations has reached values over 9,000.

TESTING PROCEDURE FOR INHIBITORS

Each substance under test was dissolved in a known concentration in human plasma. The coagulase titer of a staphylocoagulase solution was then deter-

TABLE 1

Results of inhibition tests on staphylocoagulase

SUBSTANCE TESTED	FINAL CONCENTRATION PER ML	COAGULASE TITER	
		Test	Control
Tyrothricin in propylene glycol	0.05 mg 0.055 ml	16	256
Propylene glycol	0.055 ml	32	256
Propylene glycol	0.25 ml	8	256
Gramicidin in propylene glycol	0.1 mg 0.1 ml	8	256
Tyrocidin in propylene glycol	0.1 mg 0.1 ml	8	256
Propylene glycol	0.1 ml	8	256
Gramicidin in propylene glycol	0.01 mg 0.01 ml	32	64
Tyrocidin in propylene glycol	0.01 mg 0.01 ml	32	64
Propylene glycol	0.01 ml	32	64
Penicillin G	12,500 units	512	256
Streptomycin	62.5 mg	2	512
Bacitracin	250 units	256	256
Bacitracin	50 units	128	256
Hydrazine sulfate	5.2 mg	256	128
Sulfadiazine	2.5 mg	256	256
Sulfathiazole	2.5 mg	512	256
Sodium azide	1 mg	8	32
Sodium azide	0.33 mg	16	32
Sodium azide	0.1 mg	32	32
Zephiran	0.25 mg	128	128

mined simultaneously using the same plasma as a control substrate, and the plasma plus inhibitor as the test substrate. The results of tests performed are shown in table 1.

SUMMARY

Concentrates of coagulase (prostaphylocoagulase) have been made from culture filtrates of *Staphylococcus aureus* in which the coagulase titer per milligram of nitrogen has been increased over a thousandfold. The coagulant activity of such partially purified staphylocoagulase has been found to be inhibited by streptomycin, propylene glycol, and sodium azide. It is not inhibited by penicillin, zephiran, bacitracin, tyrothricin, gramicidin, tyrocidin, hydrazine, sulfathiazole, or sulfadiazine.

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THE EFFECTS OF LYSOLECITHIN ON THE GROWTH OF LACTOBACILLUS CASEI IN RELATION TO BIOTIN, PANTOTHENIC ACID, AND FAT-SOLUBLE MATERIALS WITH BIOTIN ACTIVITY

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Williams and Fieger (1946) found that *Lactobacillus casei* could be grown continuously in a medium containing only traces of biotin if oleic acid was present at a suitable concentration. Trager (1947) observed a similar effect with a neutral oil (designated FSF) obtained from hydrolyzed horse plasma. This material, which had some biotin activity for chickens as well as for bacteria, permitted full growth of *L. casei* even in the presence of enough fresh egg white to inactivate completely any traces of biotin which might have been present in the medium. These results have been confirmed and extended (Williams and Fieger, 1947; Axelrod, Hofmann, and Daubert, 1947; Hofmann and Axelrod, 1947; Williams, Broquist, and Snell, 1947).

Lecithin exerts growth-stimulating effects on the lactic acid bacteria (Bauernfeind, Sotier, and Bowff, 1942; Strong and Carpenter, 1942) and can replace oleic acid for organisms requiring this material (Williams, Broquist, and Snell, 1947). It can also replace biotin in the growth of *L. casei* (Trager, unpublished). Since the effects of lecithin may be supposed to be a result of its oleic acid content, it seemed of interest to investigate the effects of lysolecithin, which differs from lecithin in that it does not contain an unsaturated fatty acid.

MATERIALS AND METHODS

Two samples of lysolecithin, both obtained from the Levene collection of the Rockefeller Institute, have been used. One, no. 1070, contained 3 to 5 per cent amino nitrogen, whereas the other, no. 1072, contained 5 to 10 per cent amino nitrogen. A stock solution of each, containing 2 mg per ml, was prepared in phosphate buffer of pH 7.4 and stored in the refrigerator. Preparation 1070 gave complete hemolysis of washed sheep red blood cells down to a concentration of 1:10,000, whereas preparation 1072 gave complete hemolysis at concentrations down to 1:50,000. The two preparations were, however, identical in their effects on the growth of *L. casei*. Since more of preparation 1070 was available, it was used for most of the experiments.

Stock cultures of *L. casei* were carried by weekly transfer in a medium consisting of 1 per cent yeast extract, 1 per cent glucose, 0.5 per cent peptone, and 1.5 per cent agar. The synthetic media used for the experiments and the method of inoculation were essentially those of Landy and Dicken (1942) slightly modified (Trager, 1947). The experimental cultures were incubated at 37 C for 4 days. Growth was measured by titration with 0.1 N sodium hydroxide.

RESULTS AND DISCUSSION

In a medium containing suboptimal amounts of biotin but an excess of all the other essential growth factors, lysolecithin inhibits the growth of *Lactobacillus casei*. Increasing the concentration of biotin counteracts the inhibitory effect. The results of a typical experiment are shown in figure 1. Lysolecithin and biotin, though structurally unrelated, behave toward each other like competitive metabolites, at least so far as the growth of *L. casei* is concerned. A number of instances of competition of this type between structurally dissimilar compounds have been previously reported (Woolley, 1947). It is possible to calculate a molar inhibition index (ratio of moles of inhibitor [lysolecithin] to moles of metab-
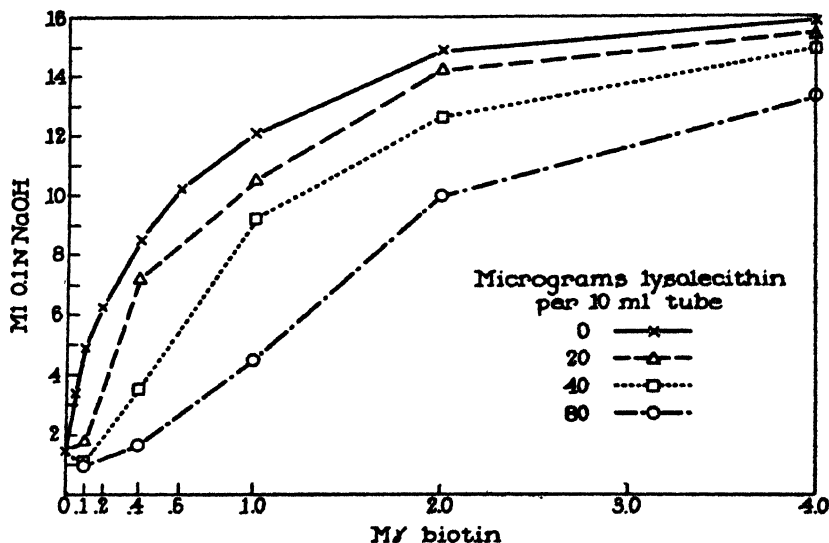
Figure 1. The inhibitory effect of lysolecithin on the growth of *Lactobacillus casei* in the presence of different concentrations of biotin.

olite [biotin] which will just prevent growth) for the antagonism between lysolecithin and biotin. This index has varied in different experiments between 50,000 and 100,000. For example, in the experiment shown in figure 1, 20 μg of lysolecithin just prevented growth in the presence of 0.1 mμg of biotin, while 80 μg of lysolecithin were effective against 0.4 mμg of biotin. In both cases the molar inhibition ratio is 100,000. In another experiment almost complete inhibition of growth was obtained by 16 μg of lysolecithin in the presence of 0.1 mμg of biotin and by 30 μg in the presence of 0.2 mμg of biotin, the molar inhibition index being 75,000. However, if the biotin of the medium was replaced by various appropriate concentrations of either oleic acid (USP) or FSF from hydrolyzed horse plasma, lysolecithin had a small growth-stimulating effect. This result is well illustrated by the experiment shown in figure 2. Note that whereas 50 or 60 μg of lysolecithin almost completely prevented growth in the presence

of 0.3 mμg of biotin, the same concentration or even ten times as high a concentration of lysolecithin stimulated growth in the presence of concentrations of FSF or oleic acid of roughly similar biotin activity. This experiment incidentally illustrates the fact that although FSF, like certain esters of oleic acid (Williams and Fieger, 1947), gives a response curve which almost parallels that with biotin, oleic acid gives an appreciably lower curve, probably because of its greater toxic-
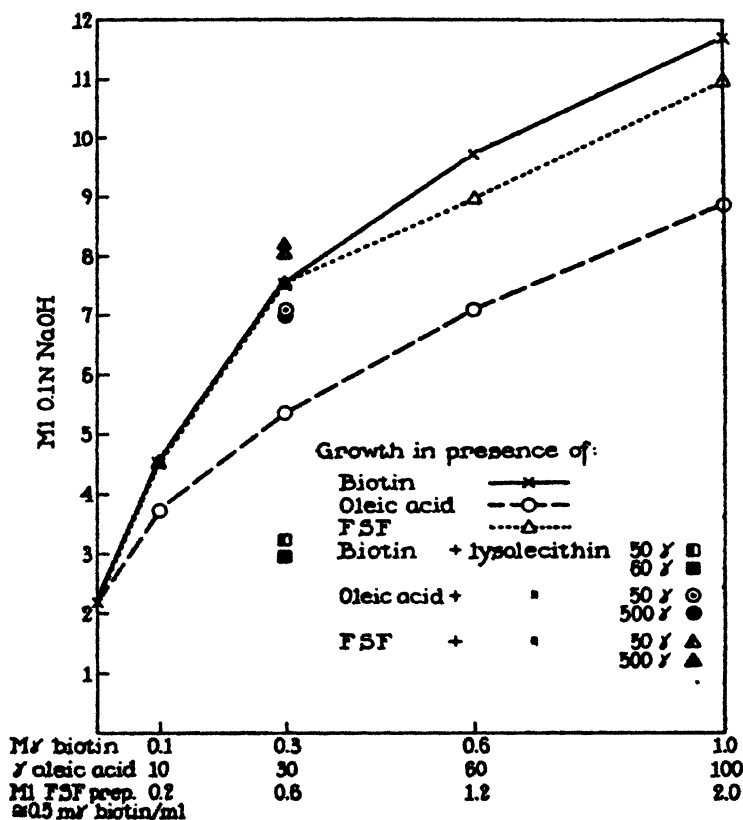
Figure 2. The growth response of *Lactobacillus casei* to biotin, oleic acid, and FSF, and the effect of lysolecithin in the presence of each of these.

ity. The stimulatory effect of lysolecithin in the presence of FSF or oleic acid may be the result of a detoxification similar to that described for lecithin and some other materials (Kodicek and Worden, 1945).

Although oleic acid and certain other fatty compounds can fully replace biotin in the nutrition of some of the lactobacilli, they cannot replace riboflavin or pantothenic acid. In the absence of either of these growth factors, or in the presence of optimal concentrations of both, oleic acid and related materials have no growth-stimulating effect. But in the presence of suboptimal concentrations of either, they have a marked effect (Bauernfeind, Sotier, and Bowff, 1942; Strong

and Carpenter, 1942). It was therefore not surprising to find that lysolecithin behaved toward calcium pantothenate, as well as toward biotin, as a competitive inhibitor. This could be clearly shown by using a medium with an excess of biotin and graded concentrations of calcium pantothenate and lysolecithin. In this case the molar inhibition index was only 500 to 1,000. If the biotin of the medium was replaced by either FSF or oleic acid at optimal concentration (an excess could not be used since the higher concentrations of these materials are
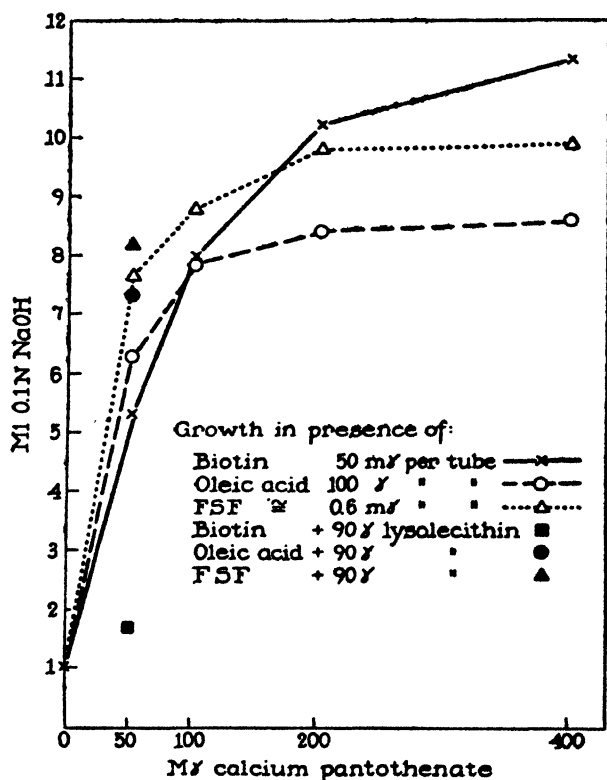
Figure 3. The growth response of *Lactobacillus casei* to calcium pantothenate in media containing biotin or, in place of it, either oleic acid or FSF, and the effect of lysolecithin in each of these media.

toxic), the response curve to graded amounts of pantothenate was much steeper. Moreover, under these conditions, lysolecithin had, not an inhibitory effect, but a slight stimulatory one. These results are shown by the experiment of figure 3.

Any attempt at a complete explanation of the phenomena described in the present paper must await a better understanding of the relationship between biotin and the fatty materials which can replace it in the nutrition of certain organisms. It seems most reasonable to suppose that biotin functions in the synthesis of these fatty materials (Williams, Broquist, and Snell, 1947). Lyso-lecithin might then behave as a true competitive analogue and in some manner

block the synthesis.   It could have no such effect if the products of the synthesis were supplied in the medium.   In the light of the results with pantothenate and lysolecithin it would be necessary to extend the argument to assume that pantothenate also functions in the synthesis of the fatty materials.   In any case it is interesting to note that lysolecithin has been observed to have a deleterious effect when fed to rats (Iwata, 1934).   This effect was prevented by the inclusion of 3 per cent yeast in the diet.

SUMMARY

The growth of *Lactobacillus casei* could be completely prevented by the addition of low concentrations of lysolecithin to a medium which was complete except that it contained biotin in suboptimal amounts.   With biotin concentrations up to 1 mμg per tube, the amount of lysolecithin required to give complete inhibition varied directly with the concentration of biotin.   If oleic acid or a fat-soluble biotin-active material from plasma was used in place of biotin, lysolecithin at concentrations up to ten times those found inhibitory with biotin had only a small growth-stimulating effect.   In a medium containing excess biotin but suboptimal concentrations of pantothenic acid, growth of the organism was inhibited by appropriate concentrations of lysolecithin.   Here again, if the biotin was replaced by an adequate concentration of oleic acid or the fat-soluble material from plasma, lysolecithin had a slight stimulatory effect rather than an inhibitory one.

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THE SERIAL PASSAGE OF MIXTURES OF DIFFERENT STRAINS OF INFLUENZA VIRUS IN EMBRYONATED EGGS AND IN MICE[1]

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There is no method available, analogous to the plating of bacteria, by means of which "cultures" of influenza virus derived from a single viral particle may be obtained. Therefore, the "purity" of some strains of that virus may depend upon the effectiveness of the usual methods of cultivation in separating mixtures of antigenically different strains.

It is evident from studies on the interference phenomenon that different strains can multiply simultaneously in tissue cultures (Andrewes, 1942) and in embryonated eggs (Ziegler and Horsfall, 1944). But whether or not such mixtures would persist if transferred in series is not evident from the literature. In order to obtain information of that sort, attempts were made to maintain mixtures of influenza A and influenza B viruses by serial passage in embryonated eggs and in mice. The present paper reports the results of those experiments.

MATERIALS AND METHODS

Viruses. The PR8 and CC strains of influenza A virus and the Lee strain of influenza B were used in the study.

Egg-to-egg passage. Three eggs containing 10- or 11-day-old embryos were used for each passage. The material inoculated was allantoic fluid diluted 10^{-3} in sterile infusion broth to which sufficient penicillin had been added to provide 50 units per inoculated dose. The volume of the inoculum was always 0.15 ml and was introduced into the allantoic sac. The inoculated eggs were incubated for 55 to 65 hours at 35 C, and were chilled before the allantoic fluids were harvested. Passage was made with the fluid in which the ratio of influenza B to influenza A virus was nearest unity, as determined by agglutination-inhibition tests.

Identification of virus in infected allantoic fluid. The identity and concentration of the virus or viruses present in the allantoic fluids were determined by agglutination inhibition tests. Serial 2-fold dilutions of each fluid were made in triplicate in 0.2-ml quantities; to each tube of one series were added 0.2 ml of anti-influenza serum (A or B), or saline, and 0.2 ml of a 1 per cent suspension of chicken erythrocytes. The contents of the tubes were mixed and the pattern of the sedimented cells was noted after 1 hour at room temperature. The antiserums were used in an initial dilution of 1:75; in that dilution they were sufficiently potent to inhibit agglutination by low dilutions of the homologous virus but had little effect upon the capacity of the heterologous virus to agglutinate the cells.

Mouse-to-mouse passage. For passage, approximately 0.07 ml of a 2.5 per cent suspension of infected mouse lung were given intranasally to each of 4 Swiss mice (3 to 5 weeks old) under ether anesthesia. The lungs of one mouse, which either had died or was sacrificed on the fourth day after inoculation, were ground in sufficient sterile distilled water to make a 10 per cent suspension. The suspension was clarified by centrifugation, and a portion was further diluted with distilled water to a final concentration of 2.5 per cent for use as passage material. A measured amount of the 10 per cent suspension was stored in the CO_2 ice chest for later determination of the identity of the virus or viruses present.

Identification of virus in infected mouse lungs. The identity of the virus or viruses present in the infected mouse lungs was determined by means of mouse protection tests, in which serial 3-fold dilutions of lung suspension (prepared in sterile distilled water containing 10 per cent normal horse serum) were tested against constant amounts of antiserums. The anti-CC and anti-Lee serums were used in an initial dilution of 1:10; the anti-CC + anti-Lee serum was prepared by mixing equal volumes of 1:5 dilutions of these two serums (i.e., the mixtures contained a 1:10 dilution of each serum). The tests were terminated on the tenth day.

EXPERIMENTAL RESULTS

Serial passage of a mixture of PR8 and Lee viruses in embryonated eggs. The original inoculations were made with a mixture of freshly harvested allantoic fluids obtained from eggs inoculated 48 hours previously with either PR8 or Lee virus. The fluids were combined in proportions so that the resulting mixture contained approximately equal amounts of the two viruses, as determined by their capacities to agglutinate chicken erythrocytes. Nine egg-to-egg passages were made as described under Methods. Since the allantoic fluids from all the eggs of any one passage were similar in their agglutinating capacities, only the results of the tests with the fluids used for inoculation are presented (table 1).

The data in table 1 show that all the allantoic fluids agglutinated the erythrocytes in the presence of either anti-PR8 or anti-Lee serum. If that agglutination is evidence of the presence of the Lee and PR8 viruses, both strains not only were detected in each fluid but had multiplied during each passage, since the small amounts of inoculated viruses (0.15 ml of a 10^{-3} dilution) were insufficient to cause the hemagglutination. Although, in some fluids, the concentration of Lee virus was lower than was the concentration of PR8, it was not an indication that the Lee was being eliminated. That is evident from the fact that the allantoic fluids from the eggs used for the eighth passage had significantly higher Lee:PR8 ratios than did the material (seventh passage fluid) with which those eggs had been inoculated. Thus, it seems quite possible that the Lee-PR8 mixture might have been maintained indefinitely by the methods of cultivation used in the experiment.

Influence of strain of influenza A virus used in mixtures upon survival of Lee virus during serial passage in embryonated eggs. Results quite different from those reported in the previous section were obtained from experiments in which

TABLE 1

*The persistence of both PR8 and Lee viruses during
serial passage in embryonated eggs*

PASSAGE NO.	HEMAGGLUTINATION[*] BY MIXTURES OF DILUTIONS OF ALLANTOIC FLUIDS AND CONSTANT AMOUNTS OF ANTISERUM										
		Initial 2-fold dilution of allantoic fluid									
	Serum	1	2	3	4	5	6	7	8	9	10
0	Anti-PR8	++	++	++	++	++	++	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	0	0	0	0
	Saline	++	++	++	++	++	++	++	+	0	0
1	Anti-PR8	++	++	++	++	++	0	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	+	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
2	Anti-PR8	++	++	++	++	++	++	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	++	+	0	0
	Saline	++	++	++	++	++	++	++	++	+	0
3	Anti-PR8	++	++	++	++	++	++	+	0	0	0
	Anti-Lee	++	++	++	++	++	++	+	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
4	Anti-PR8	++	++	++	++	++	++	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	0	0	0	0
	Saline	++	++	++	++	++	++	++	0	0	0
5	Anti-PR8	++	++	++	++	++	++	+	+	0	0
	Anti-Lee	++	++	++	++	++	++	++	++	0	0
	Saline	++	++	++	++	++	++	++	++	+	0
6	Anti-PR8	++	++	++	++	0	0	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	++	++	+	0
	Saline	++	++	++	++	++	++	++	++	++	0
7	Anti-PR8	++	++	++	0	0	0	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	++	++	+	0
	Saline	++	++	++	++	++	++	++	++	++	0
8	Anti-PR8	++	++	++	++	++	+	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	++	++	++	0
	Saline	++	++	++	++	++	++	++	++	++	0
9	Anti-PR8	++	++	+	0	0	0	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	++	++	0	0
	Saline	++	++	++	++	++	++	++	++	0	0

[*] ++, +, and 0 = complete, partial, and no agglutination, respectively.

the starting material consisted of a mixture of the CC strain of influenza A virus
and the Lee strain of influenza B virus. The infected allantoic fluids were com-

bined in proportions so that the Lee:CC ratio varied, in different experiments, from 1 to 8 as measured by their hemagglutinating capacities. In all the experiments the allantoic fluids obtained from eggs inoculated with the original ma-

TABLE 2

The elimination of Lee virus from a mixture of CC and Lee viruses during serial passage in embryonated eggs

PASSAGE NO.	Serum	HEMAGGLUTINATION* BY MIXTURES OF DILUTIONS OF ALLANTOIC FLUIDS AND CONSTANT AMOUNTS OF ANTISERUM									
		Initial 2-fold dilution of allantoic fluid									
		1	2	3	4	5	6	7	8	9	10
0	Anti-CC	++	++	++	++	++	++	++	+	0	0
	Anti-Lee	++	++	++	++	++	++	+	0	0	0
	Saline	++	++	++	++	++	++	++	++	0	0
1	Anti-CC	++	++	++	++	++	0	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	++	++	0	0
	Saline	++	++	++	++	++	++	++	++	++	0
2	Anti-CC	0	0	0	0	0	0	0	0	0	0
	Anti-Lee	++	++	++	++	++	++	++	++	0	0
	Saline	++	++	++	++	++	++	++	++	+	0

* ++, +, and 0 = complete, partial, and no agglutination, respectively.

TABLE 3

The persistence of both CC and Lee viruses during serial passage in mice

PASSAGE NO.	Anti-CC + anti-Lee			Anti-CC					Anti-Lee					
	THE NUMBER OF DEATHS* FOLLOWING INOCULATION OF DECREASING CONCENTRATIONS OF LUNG SUSPENSIONS PLUS CONSTANT AMOUNT OF SERUMS													
	Serial 3-fold dilutions of 5% lung suspension													
	0	1	2	0	1	2	3	4	0	1	2	3	4	5
0	0	0	0	3	3	1	0	0	3	3	2	0	0	0
1	0	0	0	3	3	0	0	0	3	3	3	3	3	0
2	0	0	0	3	3	3	0	0	3	3	3	3	3	2
3	0	0	0	3	3	3	2	0	3	3	3	3	3	1
4	0	0	0	3	3	3	1	0	3	3	3	3	1	0
5	0	0	0	3	3	2	2	1	3	3	3	3	3	2
6	0	0	0	0	0	0	0	0	3	3	3	3	3	0
7	0	0	0	0	0	0	0	0	3	3	3	3	3	2

* Three mice were inoculated with each serum-virus mixture.

terial contained both A and B viruses. But in no instance was Lee detectable by agglutination inhibition tests in the fluids from the second passage. The results of one of those experiments are shown in table 2.

A comparison of these data (table 2) with those contained in table 1 emphasizes that the experimental results may vary with different strains of influenza virus, even though the strains are closely related antigenically.

Serial passage of a mixture of CC and Lee viruses in mice. The CC and Lee strains of influenza virus were selected for use in the mouse transfer experiments because it was desired to include in the starting material an A and a B virus which possessed similar pathogenic properties for those animals. Of the mouse-adapted strains that had been extensively studied in this laboratory,, those two strains seemed most nearly to meet that requirement.

The original inoculations were made with material prepared by combining a suspension of lungs from mice that had recently died from infection with CC with like material from mice that had died from infection with Lee. Seven mouse-to-mouse transfers were made, and the identity of the virus or viruses present in the infected lungs was determined as described under Methods. The results of the tests are shown in table 3.

It is evident (table 3) that after 5 mouse-to-mouse transfers both CC and Lee virus were present in the lung suspension in as high concentrations as had been observed in any of the previous tests. It also is evident that there was a sudden drop in the concentration of Lee during the sixth passage. However, the Lee virus was not completely eliminated. Although it is not shown in the table, the mice inoculated with mixtures of anti-cc serum and low dilutions of test material from both the sixth and the seventh passages showed fairly extensive lung lesions when sacrificed on the tenth day of the test. In contrast, mice inoculated with mixtures of anti-CC + anti-Lee serum and the same dilutions of the same test materials did not show such lesions. The fact that the CC-Lee mixture was maintained for at least 5, and probably 7, mouse-to-mouse transfers, seems to be of significance. It suggests that some influenza viruses might be transferred as a mixture, in that species, for a long period of time, if not indefinitely.

DISCUSSION

The results of the present experiments have demonstrated that some mixtures, composed of influenza A and influenza B viruses, could be passed in series in embryonated eggs and in mice. It would seem even more likely that a combination of strains more closely related than the ones used in these experiments might be maintained as a mixture for an indefinite number of transfers. In that event the mixture would be designated as a "strain." The antigenic pattern of such a "strain" would be a composite of antigenically different particles and a shift in the relative proportions of the component antigenically different particles would be reflected as a change in the antigenic composition of the "strain." That such a shift can occur following propagation in different host species might be indicated by the results of the present experiments with the CC and Lee viruses; although the same starting material was not used for the two experiments, those viruses were maintained as a mixture for at least 5, and probably 7, mouse-to-mouse transfers (table 3), but the CC virus quickly eliminated the Lee from the mixture during serial passage in eggs (table 2).

It is possible that most if not all of the strains of influenza virus are antigenically "pure." But, it also is possible that in some instances the materials from which the various influenza viruses have been obtained may have contained

antigenically different strains, particularly if the starting material consisted of pooled specimens from two or more patients, because it has been shown (Magill and Sugg, 1943) that antigenically different strains may occur in the same outbreak. In addition, the danger of laboratory contamination is always present. Therefore, it would seem highly desirable that more definite evidence of "purity" be obtained, especially in the case of those strains used for experiments on variation.

The successful transfer of the PR8-Lee mixture in embryonated eggs is of additional interest in view of the results of experiments published by Henle, Henle, and Rosenberg (1947). Those authors reported that the interval between the inoculation of virus into the allantoic sac and the first release of virus by the infected cells was a little more than 6 hours in the case of PR8, but was a little more than 9 hours in the case of Lee. The shorter "growth cycle" for PR8 suggests that Lee virus should have been speedily eliminated from the PR8-Lee mixture. But in our experiments (table 1) the Lee virus was not eliminated, even after 9 egg-to-egg transfers. Thus, one might question whether or not the PR8 and the Lee strains used in Henle's laboratory were identical with the PR8 and the Lee strains used in our laboratory.

SUMMARY

It was found that mixtures of some strains of influenza A and influenza B viruses could be passaged in series in embryonated eggs and in mice. In view of those results, it would seem likely that a combination of more closely related strains might be maintained indefinitely by the usual methods of laboratory cultivation. The data, therefore, raise the question of whether or not all strains of influenza virus are antigenically "pure," and indicate the desirability of obtaining more definite evidence of "purity" in the case of strains to be used in experiments on variation.

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OBSERVATIONS ON BACTERIA SENSITIVE TO, RESISTANT TO, AND DEPENDENT UPON STREPTOMYCIN[1,2]

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Streptomycin resistance is usually manifested by growth either in the presence of streptomycin or in its absence. Resistant variants generally are similar, morphologically and biochemically, to the parent sensitive strain (Miller and Bohnoff, 1946; Murray *et al.*, 1946). Some differences, such as alteration in motility and changes in growth rate and in biochemical reactions, have been reported, but these have been inconsistent (Graessle and Frost, 1946; Klein and Kimmelman, 1946; Murray *et al.*, 1946; Petroff and Lucas, 1946; Seligmann and Wassermann, 1947; Stubblefield, 1947). Streptomycin-resistant organisms have ordinarily been obtained *in vitro* by repeated subculture in increasing concentrations of this antibiotic (Chandler and Schoenbach, 1947; Miller and Bohnhoff, 1946; Murray *et al.*, 1946; Price *et al.*, 1947; Seligmann and Wassermann, 1947), though the occurrence of a few very resistant organisms in a large inoculum of sensitive ones on initial exposure to streptomycin has been described in the case of *Shigella* (Klein, 1947), *Escherichia coli* (Clark and Rantz, 1947; Klein, 1947), *Staphylococcus aureus* (Klein, 1947), *Staphylococcus albus* (Klein, 1947), *Proteus vulgaris* (Klein, 1947), *Hemophilus influenzae* (Alexander and Leidy, 1947), meningococcus (Miller and Bohnhoff, 1947a), and *Mycobacterium tuberculosis* (Vennesland, Ebert, and Bloch, 1947).

In the case of the meningococcus, Miller and Bohnhoff (1947a,b) described another type of resistant variant which apparently required streptomycin for growth. More recently a variant of *Bacillus subtilis* which requires streptomycin for growth has been reported (Kushnick *et al.*, 1947). A possible stimulating effect of streptomycin on growth has been noted in the case of streptomycin-resistant strains of *Brucella* (Hall and Spink, 1947), *Escherichia coli* and *Pseudomonas aeruginosa* (Kushnick *et al.*, 1947), and tubercle bacilli (Vennesland, Ebert, and Bloch, 1947). An increased mortality rate of mice infected with typhoid bacilli and treated with small doses of streptomycin has been reported (Welch, Price, and Randall, 1946). Suitable low concentrations of penicillin and streptomycin stimulated growth of many sporeformers, especially *Bacillus megatherium*, and also *S. aureus* and *Streptococcus agalactiae* (Curran and Evans, 1947).

In a preliminary report from this laboratory (Paine and Finland, 1948) the derivation of streptomycin-resistant and streptomycin-dependent variants from several streptomycin-sensitive species of bacteria was noted. Further details of this investigation are given here.

<div align="center">MATERIALS AND METHODS</div>

Definition. The parent strains used to obtain the variants under study were considered to be "sensitive" to streptomycin. Their growth was completely inhibited by concentrations of 100 μg or less of streptomycin per ml of broth in the usual tube dilution test for sensitivity. "Resistant" strains were those that multiplied equally well in the presence of high concentrations of streptomycin and in its absence. "Dependent" strains were those that multiplied only in the presence of streptomycin; the amount that was required varied with the different organisms.

Media and antibiotics. The broth used throughout these studies was brain-heart infusion broth, pH 7.2 (Difco), and the agar was heart infusion agar (Difco). Various lots of streptomycin hydrochloride (Merck) and streptomycin calcium chloride complex (Merck) as available commercially were used, the amounts being recorded in terms of the streptomycin base. Crystalline penicillin G, potassium salt, was obtained from Commercial Solvents Corporation. The bacitracin was a crystalline preparation provided by Dr. Frank L. Meleney and contained 5,000 units (Johnson, Anker, and Meleney, 1945) per vial. A purified preparation of polymyxin containing 1,800 *E. coli* units (Stansly, Shepherd, and White, 1947) per mg was provided by Dr. Y. SubbaRow of the Lederle Laboratories.

Isolation of resistant and dependent variants. Each organism, previously unexposed to streptomycin, was grown overnight in broth; 10 ml of the culture were then centrifuged at 2,000 rpm for 30 minutes, the supernatant was decanted, and the sediment was suspended in 0.6 ml of saline. One-tenth ml of this suspension was used for bacterial counts in agar pour plates, and the rest was spread evenly over the surface of an agar plate containing 1,000 μg of streptomycin per ml of agar. The plate was incubated, without inverting, at 37 C for 3 to 5 days, and any colonies which appeared in that time were subcultured on plain agar plates and on plates containing graded concentrations of streptomycin.

Growth curves. A series of tubes containing graded concentrations of streptomycin in 10-ml amounts of broth were each inoculated with 0.1 ml of a 10^{-4} dilution of an overnight broth culture. The dependent variant was always grown in broth containing 1,000 μg of streptomycin per ml prior to its inoculation into the growth curve tubes. The tubes were incubated at 37 C, and aliquots were removed at varying intervals for agar pour plate counts of viable bacteria. Counts of the dependent variants were made both in streptomycin-free agar and in agar containing 1,000 μg of streptomycin per ml.

Sensitivity determinations. The sensitivity of these bacteria to antibiotics was tested by a tube dilution method (Finland *et al.*, 1946) utilizing 0.5-ml amounts of serial broth dilutions of the antibiotic to which were added similar volumes of

a 10^{-4} dilution of an overnight broth culture of the test organism (usually containing 50,000 to 100,000 organisms). The sensitivity, in each instance, was defined as the minimum concentration in which no growth occurred after 24 to 48 hours' incubation at 37 C in the antibiotic-containing broth and after another subculture for 24 hours on antibiotic-free agar. Tests with the dependent variants were always conducted in media containing 1,000 μg of streptomycin per ml.

Biochemical reactions. Fermentation tests on various sugars and other biochemical tests were made on all strains. For the dependent variants, 1,000 μg of streptomycin per ml were included in all the test materials.

RESULTS

Isolation of variants. Streptomycin-resistant and streptomycin-dependent variants were isolated from sensitive strains of *Staphylococcus aureus*, *Escherichia coli*, *Proteus morgani*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The incidence of resistant and dependent variants in these bacterial populations which had not been previously exposed to streptomycin ranged from 1 in 2 billion to 1 in 400 billion organisms. The readiness with which these variants appeared differed considerably; they were obtained quite regularly from 2 strains each of *S. aureus*, *P. morgani*, and *K. pneumoniae*, but only from 1 of 4 strains of *E. coli* and from 1 of 3 strains of *P. aeruginosa*. A statistically valid assay of the incidence of resistant and dependent variants is not possible on the basis of the number of experiments conducted thus far.

Following an initial exposure of an apparently sensitive strain to 1,000 μg of streptomycin per ml of agar, some colonies appeared which on subculture proved to consist entirely of highly resistant organisms. These organisms remained resistant on subsequent subcultures both in the presence and in the absence of streptomycin.

Evolution of the dependent variants occurred in a different manner. The "dependent" potentialities of a single colony developing from the initial exposure of a sensitive strain to a high concentration of streptomycin was determined by subculturing a saline suspension of the entire colony on a series of agar plates containing graded concentrations of streptomycin. If the colony contained dependent organisms, there was a predominance of growth on the agar plates with the higher concentrations of streptomycin. However, there was usually slight growth in the lower concentrations of streptomycin or even in its absence. After one or more subcultures from the plates containing the higher concentrations of streptomycin in which growth had been most vigorous, it was possible to obtain strains that would not grow in the absence of streptomycin, that is, "pure" dependent strains.

The nature of the organisms in the predominantly dependent colonies which grew, though sparsely, in the lower concentrations or in the absence of streptomycin was of interest. On subculture, these organisms behaved as predominantly sensitive bacteria. Thus, isolation of "pure" dependent strains involved selective subculture using the higher streptomycin concentrations in which growth had occurred, in order to eliminate the sensitive organisms with which

the dependent variants were associated. A protocol showing the derivation of *S. aureus* variants is given in table 1.

TABLE 1

Derivation of variants of Staphylococcus aureus

COLONY	CONCENTRATION OF STREPTOMYCIN, µG PER ML OF AGAR						
	1,000	100	25	6.3	3.1	1.5	0
Initial subculture							
S1	+++[a]	+++	+++	+++	+++	+++	+++[b]
S2	+++[a]	+++	+++	+	+	6	3[b, c]
S3	+++[a]	+++	+++	+	6	4	3[b]
Second subculture							
S1a	+++	+++	+++	+++	+++	+++	+++
S1b	+++	+++	+++	+++	+++	+++	+++
S2a	+++	+++	+++	+	0	0	0
S2b	3[d]	+	++	+++	+++	+++	+++[e]
S2c	1[f]	+	++	+++	+++	+++	+++[g]
S3a	+++	+++	+++	+	0	0	0
S3b	0	5[c]	+	+++	+++	+++	+++[d]
Third subculture							
S2d	+++	+++	+++	1	0	0	0
S2e	1[h]	5	++	+++	+++	+++	+++[j]
S2f	+++	+++	+++	++	++	+	0
S2g	0	0	+	+++	+++	+++	+++
S3c	0	+	++	++	+++	+++	+++
S3d	0	3	+	++	+++	+++	+++[f]
Fourth subculture							
S2h	+++	+++	+++	+++	+++	+++	+++
S2j	0	5	++	+++	+++	+++	+++
S3f	0	0	6	+++	+++	+++	+++

Each plate was inoculated with a 2-mm loopful of an emulsion of a single colony in 0.2 ml of saline.

The colonies S1, S2, and S3 were picked from an agar plate containing 1,000 µg of streptomycin per ml of agar on which the parent sensitive strain was exposed to streptomycin for the first time.

The letters indicate colonies picked from the different plates.

+++, ++, +, and 0 indicate amount of growth on the surface. When only a few isolated colonies appeared, their numbers are indicated.

Dependence upon streptomycin was not nearly so permanent a characteristic as that of resistance to the antibiotic. When grown in suboptimal concentrations of streptomycin, organisms rapidly reverted from the "dependent" to the sensitive and sometimes to the resistant state.

Colony characteristics and morphology. When grown under optimal conditions,

there were usually no differences in the colonial appearance of the sensitive, resistant, and dependent strains of the same organism. In the case of *Proteus*, however, the resistant and dependent variants showed less tendency to swarm than did the parent sensitive strains.

Microscopically, the sensitive strains showed only slight alteration from the normal appearance when grown in sublethal amounts of streptomycin. This was also true of the resistant variants grown either in the presence or in the absence of the antibiotic. On the other hand, the dependent variants showed marked pleomorphism when cultured in suboptimal concentrations of streptomycin. The dependent *Staphylococcus*, under these circumstances, exhibited great variation in the size of the cocci, some appearing greatly enlarged. Dependent
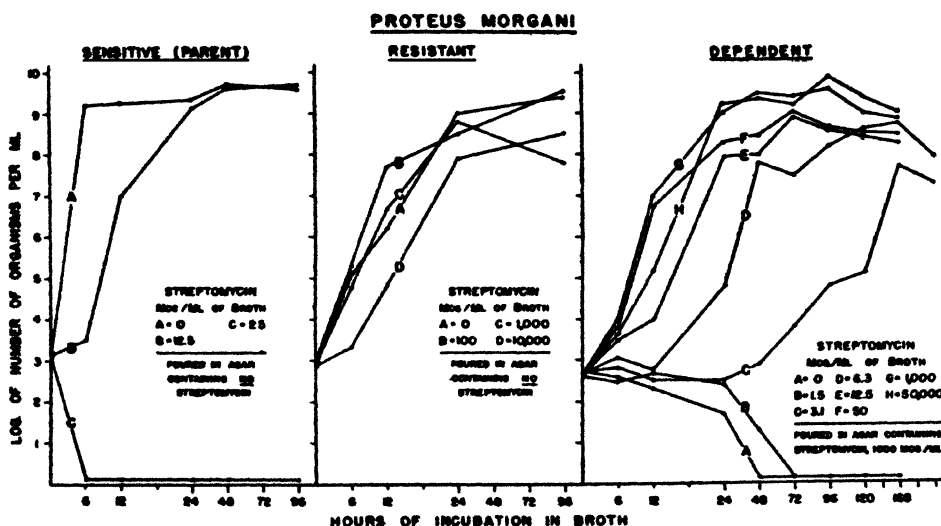
Figure 1. Representative growth curves of a sensitive strain of *P. morgani* and its resistant and dependent variants in broth containing graded concentrations of streptomycin.

variants of gram-negative bacilli grown in suboptimal amounts of streptomycin showed long filamentous structures. This was particularly striking in the case of the dependent *Proteus*, which also showed the presence of bulbous, fusiform, and densely stained swellings along the shafts of some of the filaments. No such forms were noted when any of the dependent variants were grown in optimal concentrations of streptomycin.

Growth curves. These were conducted with all of the organisms and their variants. There were several characteristics common to the variants of all of the different organisms studied. The curves for the *Proteus* variants depicted in figures 1 and 2 will serve as examples.

Sensitive strains grown in sublethal amounts of streptomycin showed a progressive increase in the lag period as the concentration of the antibiotic was increased. Though not shown in figure 1, this progression was demonstrable

with graded concentrations below 12.5 µg per ml of streptomycin in the case of the sensitive *Proteus*.

The resistant variants grew equally well in plain broth and in concentrations of streptomycin up to 1,000 µg per ml. In concentrations of 10,000 to 50,000 µg per ml there was usually some inhibition of the rate of growth of the resistant strains.

Dependent variants, except in the case of *E. coli*, apparently did not multiply in the absence of streptomycin or in very low concentrations of the drug   The dependent coli in broth containing no streptomycin showed slight initial multi-
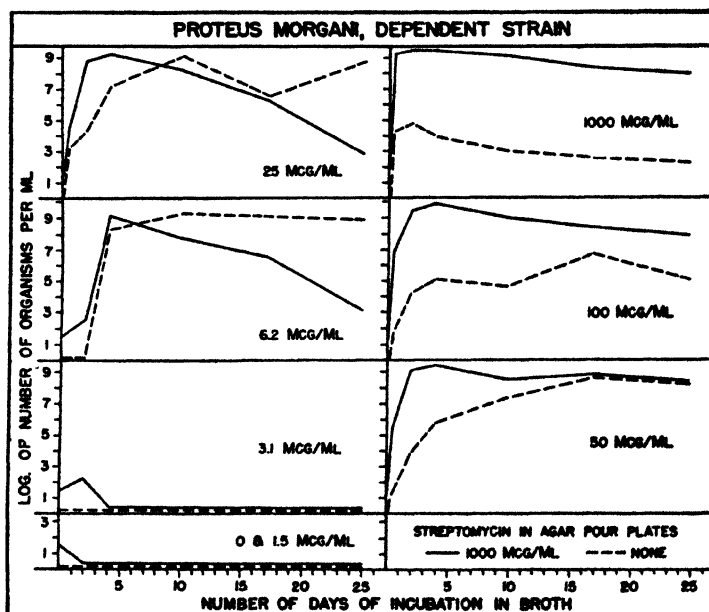
Figure 2. Growth curves of the dependent strain of *P. morgani*. Each pair of curves represents the counts from the same tube of broth poured simultaneously in agar with and without streptomycin.

plication, but growth was not maintained and the organisms soon disappeared in a fashion similar to the dependent *Proteus* shown in curve A, figure 1. All of the dependent strains showed a stepwise increase in the rate of growth, or shortening of the lag period, in increasing concentrations of streptomycin. Maximum rates of growth were achieved in the range to 500 to 1,000 µg per ml. Some inhibition of the rate of growth of the dependent variants occurred in very high concentrations of the antibiotic (curve H in figure 1). The dependent *Staphylococcus* occasionally failed to grow in a concentration of 20,000 µg of streptomycin per ml. The maximum rates of growth of both the resistant and dependent variants were usually less rapid than the maximum rate of growth of the corresponding parent sensitive strains.

Certain other properties of the dependent variants are illustrated by the curves shown in figure 2. In this instance the solid lines represent the colony counts in agar containing 1,000 µg of streptomycin per ml, and the broken lines depict colony counts from the same tube of broth poured simultaneously in agar containing no antibiotic. In each tube in which vigorous growth of the dependent organism took place organisms appeared in the pour plates both with and without streptomycin. Colonies appearing in the plain agar plates probably did not represent dependent organisms growing in the small amount of streptomycin carried over from the broth tubes, since these concentrations were inadequate for that purpose.

From the tubes containing the higher concentrations of streptomycin the colonies which appeared in the plain agar pour plates never approached in numbers those appearing simultaneously in the streptomycin agar pour plates. On the other hand, in the lower concentrations (6.2 to 50 µg per ml) the colonies which appeared in the plain agar plates usually approached in numbers those appearing at the same time in the streptomycin agar plates and sometimes exceeded them.

Studies with single colonies from plates poured after 25 days' incubation of the broth tubes showed that the organisms from the 6.2- and 25-µg-per-ml tubes were now largely sensitive bacteria; the colonies from the 50-µg-per-ml tube consisted largely of resistant organisms; whereas those from the tubes containing 100 and 1,000 µg per ml were still predominantly dependent. Thus, in the case of the dependent *Proteus* reversion from the dependent to the sensitive state occurred after prolonged incubation in the presence of 6.2 or 25 µg of streptomycin per ml, and resistant organisms emerged in the presence of 50 µg per ml.

It seems likely that vigorous growth of the dependent strains was accompanied by the appearance of sensitive variants. In general, when this occurred in the presence of high concentrations of streptomycin, further growth of the sensitive variants was suppressed; but when this took place in low concentrations of the antibiotic, multiplication of the sensitive variants was possible and either sensitive or resistant strains seemed to emerge. When vigorous growth of the dependent strains did not take place, as in the very low concentrations of streptomycin, neither sensitive nor resistant variants appeared.

Biochemical reactions. Study of the fermentation of various carbohydrates, the production of indole, ammonia, hydrogen sulfide, and acetoin, nitrate reduction, citrate utilization, liquefaction of gelatin, hemolytic and coagulase activity, pigment production, and motility disclosed no differences between the sensitive bacteria and their derived resistant and dependent variants in most instances. Two sensitive strains of *K. pneumoniae* fermented dulcitol with the production of gas, whereas their resistant and dependent variants did not ferment dulcitol. A sensitive strain of *K. pneumoniae*, type C, uniformly showed capsular swelling and clumping with homologous rabbit antiserum; its resistant variant showed capsule swelling and clumping of only some of the organisms, whereas the dependent variant exhibited neither swelling of the capsules nor clumping in the presence of the antiserum.

Sensitivity to other antibiotics. In table 2, are shown the results of sensitivity determinations conducted with the 3 variants of each of the organisms against penicillin, bacitracin, and polymyxin. There were only minor differences in the sensitivity of the three variants of each organism to these antibiotics.

Effect of streptomycin inhibitors. Cysteine hydrochloride and semicarbizide, two substances which inhibit the action of streptomycin on susceptible bacteria, also interfered with the utilization of this antibiotic by the dependent variants.

Critical concentration of streptomycin. As previously noted (Paine and Finland, 1948), growth ceased in the case of both the sensitive and dependent strains

TABLE 2

Sensitivity to other antibiotics of variants sensitive to, resistant to, and dependent upon streptomycin

ORGANISM	VARIANT	MINIMUM CONCENTRATION FOR COMPLETE INHIBITION, UNITS PER ML		
		Penicillin	Bacitracin	Polymyxin
Staphylococcus	Sensitive	0.125	1.0	>450
aureus	Resistant	0.125	1.0	>450
	Dependent	0.031	0.125	225
Escherichia	Sensitive	39	500	0.016
coli	Resistant	39	500	0.016
	Dependent	39	500	0.031
Proteus morgani	Sensitive	1,250	500	>450
	Resistant	1,250	500	>450
	Dependent	625	500	50
Pseudomonas	Sensitive	>10,000	>500	1.0
aeruginosa	Resistant	>10,000	>500	2.0
	Dependent	5,000	>500	1.0
Klebsiella	Sensitive	78	200	2.0
pneumoniae	Resistant	39	50	0.125
	Dependent	39	100	0.25

of the same organism at about the same concentration of streptomycin. This was true for *S. aureus, E. coli, P. aeruginosa,* and *P. morgani.* The same was true also of *K. pneumoniae;* growth of its parent sensitive strain was inhibited by 3.1 μg of streptomycin per ml, and this was the minimum concentration supporting growth of its dependent variant.

DISCUSSION

The most widely accepted explanation for the ability of microorganisms to survive in unfavorable concentrations of chemotherapeutic or antibiotic agents is that it results from the selection of chance mutants which can survive in that environment (Alexander and Leidy, 1947; Demerec, 1945; Klein and Kimmel-

man, 1946; Luria, 1947; Miller and Bohnhoff, 1947a,b). The present observations, like those of Miller and Bohnhoff (1947a,b) with the meningococcus, show that the ability of certain bacteria to survive exposure to an ordinarily lethal concentration of streptomycin is due to the appearance of variants which possess either of two characteristics: (1) independence of streptomycin activity, more commonly known as resistance to streptomycin, or (2) dependence upon streptomycin for growth. The first trait seems to be a relatively permanent one and apparently involves the entire progeny of the resistant organism once it appears. It thus more nearly fulfills the usual criteria of a mutant. This resistance, however, is not completely permanent in every instance since reversion to a sensitive status has been recorded in the case of certain streptomycin-resistant organisms when they were subcultured many times in the absence of the antibiotic (Murray, Wilcox, and Finland, 1947).

Dependence upon streptomycin appears to be a much more labile trait than resistance to this antibiotic. In this respect the dependent variants studied in this laboratory varied considerably from the dependent strains of meningococci described by Miller and Bohnhoff (1947b), in which there apparently was little tendency for a change from the dependent state. All of the dependent variants studied here were found, during growth in broth, to give rise to organisms which would grow in the absence of streptomycin. The evidence suggests that the dependent variants give rise only to streptomycin-sensitive organisms. The latter, in turn, if given the opportunity to multiply in sublethal amounts of streptomycin, may then give rise to resistant strains. Reversion from the dependent state took place only during multiplication of the organisms. Mutations directly from the dependent to resistant strains or the reverse may occur, but the present observations do not favor such possibilities.

The present findings suggest that dependence upon and sensitivity to streptomycin may be closely related phenomena. (1) Both sensitive and dependent organisms were demonstrated in the same "predominantly dependent" colony which appeared following the initial exposure of the sensitive strain to streptomycin. (2) The dependent variants seem to give rise only to sensitive organisms under appropriate conditions. (3) The same concentration of streptomycin which completely inhibited the growth of the parent sensitive strain seemed necessary for growth of its dependent variant. No such relationships could be demonstrated in the case of the resistant variants.

The exact mechanism by which streptomycin exerts its antibacterial effect is not known. Assuming that it is effective against sensitive organisms by blocking some essential metabolite or enzyme system, it seems to be more than mere coincidence that approximately the same concentration of streptomycin which inhibits growth of the sensitive strain becomes necessary for growth of the dependent variant of the same strain. Is the original essential metabolite now replaced by some moiety of the streptomycin molecule which can be utilized in its place? In the case of the resistant variants, they grow independently of the presence or absence of streptomycin. It would seem, therefore, that an alternative mechanism or system exists in the resistant variant which functions

in the place of the metabolite or enzyme system that is blocked by streptomycin in the sensitive strain.

Another change which appears in both the dependent and resistant variants is that they grow at a slower rate than their parent sensitive strain. This is an interesting mutational character similar to the slower growth rates described in the case of certain phage-resistant variants (Luria, 1946). This slower growth rate of the resistant variant may explain why it is not normally encountered until the more rapidly growing sensitive organisms are inhibited by streptomycin.

Consistent biochemical differences between the sensitive, dependent, and resistant variants of the same organisms were not encountered except in the case of *K. pneumoniae*. Similar changes were previously noted in resistant strains of this organism (Murray *et al.*, 1946). The differences in sensitivity to other antibiotics of some of the three variants are not explained.

The pleomorphic forms of the streptomycin-dependent variants grown in suboptimal amounts of streptomycin were similar to those observed by many workers when organisms were grown under unfavorable conditions (Dienes, 1947; Gardner, 1925, 1945; Gay and Clark, 1937; Shanahan, Eisenstark, and Tanner, 1947; Tunnicliff, 1939; Walker and Murray, 1904; Wyckoff, 1933, 1934).

Miller and Bohnhoff (1947b) have pointed out that one cannot be certain that their dependent variants were actually dependent on streptomycin itself and not on some impurity. However, other bacterial and fungal mutants have been described that have undergone marked transformations in their growth requirements. For instance, Kohn and Harris (1942) were able to derive a methionine-requiring variant of *E. coli* by subculturing the parent strain in a medium containing sulfanilamide and certain amino acids. A strain of *Neurospora* requiring sulfanilamide for growth has been described (Emerson, 1947) and stimulation of growth of *P. aeruginosa* and *Alcaligenes faecalis* by sulfanilamide has been observed (Lamanna, 1942).

SUMMARY AND CONCLUSIONS

Streptomycin-resistant and streptomycin-dependent variants have been obtained from streptomycin-sensitive strains of *Staphylococcus aureus*, *Escherichia coli*, *Proteus morgani*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

The growth characteristics, morphologic features, and certain biochemical reactions of these organisms and of their derived variants have been described.

The evidence suggests that resistance to streptomycin is a relatively permanent trait, whereas dependence upon streptomycin is a readily reversible phenomenon.

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UNIDENTIFIED NUTRIENTS IN TETANUS TOXIN PRODUCTION

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A series of reports from this laboratory has established the following: (1) The requirements for growth of a strain of *Clostridium tetani* originally obtained from the Division of Laboratories, New York State Department of Health, have been quite accurately defined (Mueller and Miller, 1942). (2) By making use of the information so obtained, the production of toxin on a "peptone-free," though by no means "synthetic," medium has been accomplished (Mueller and Miller, 1943). (3) Under these conditions the yield of toxin was sharply dependent upon the careful adjustment of Fe concentration at a very low level. This toxin, although formed in relatively poor titer, has been converted to toxoid, and the latter has been shown to be an adequate antigen in both animals and man (Mueller, Seidman, and Miller, 1943a, b). (4) Attempts at increasing the potency of the toxin by modification of the medium within the known framework of its constitution failed. (5) Substitution of a hog stomach autolysate medium (Mueller and Miller, 1945) for the peptone-free medium revealed a completely altered relationship of toxin formation to iron concentration in that high yields of toxin were obtained in the presence of an excess of Fe. (6) A tryptic digest of casein supplemented by cystine and tyrosine would replace the hog stomach autolysate (Mueller and Miller, 1947). (7) Toxin obtainable under these conditions was many times more potent than that produced on a "peptone-free" medium at low and controlled iron concentration and flocculated promptly and sharply with antitoxin at or near the neutral point. It was readily convertible to toxoid, and the latter was a satisfactory antigen (Mueller and Miller, 1945).

Maximum titers of toxin were obtained only on a small scale, i.e., in 20 ml of medium in 6-by-$\frac{3}{4}$-inch tubes, the production scale generally was 30 to 60 per cent of the level reached in smaller quantity. This may be due in part to temperature effects (Mueller and Miller, 1948), in part to differences in the degree to which gaseous products of growth (CO_2 and H_2S) diffuse away from the culture (Mueller, Miller, and Lerner, 1948), and possibly in part to other still undefined factors.

Fundamentally, our continued interest in the problem is to attempt the chemical definition of the components of the culture medium that are essential for high toxin production under the conditions specified above. A complete acid hydrolyzate of casein, supplemented by those amino acids in which it is known to be deficient, will not replace the tryptic digest. It therefore appears that one or more chemical structures or linkages which are destroyed by heating with acid must be concerned. Possibly these are peptides, and an elucidation of their nature may relate the requirements of the tetanus bacillus to those of

other forms of life, thus yielding information of general biological importance. For example, Sprince and Woolley's (1945) strepogenin may be involved. Again, the somewhat more complex requirements described by Adams and others (1947) for toxin production by *Clostridium welchii*, and the observations of Womack and Rose (1946) on enhanced growth of rats when fed the products of tryptic digests of protein, point to the existence of a nutritional phenomenon of general significance. For these reasons considerable effort has been expended in attempts designed to isolate and identify the chemical substances that are concerned in the production of high levels of tetanus toxin. Although the work is far from completion, it appears that enough information has been gained to warrant preliminary presentation, especially because of the possibility that some of the results and methods may be found useful by others concerned with similar problems.

GENERAL METHODS

A basic medium of the following composition is used as the positive control in each experiment, and this medium, with the tryptic digest of casein omitted but containing 1 mg of tryptophan, may serve as the negative control. The latter is now usually omitted since growth is invariably sparse, and no toxin is formed which is detectable by flocculation.

For 20-ml quantities of medium:

Double strength beef heart infusion (1)...	5.0 ml
Tryptic digest of casein (2)..	300 mg
Glucose....	0.15 g
Cystine (3)	2.5 mg
Tyrosine (4)....	10 mg
NaCl....	.05 g
Salts (5)...	0.5 ml
Phenol red 0.2 per cent.........	0.05 ml

(1) Minced beef heart muscle is suspended in an equal weight of water, brought to an active boil while stirring, strained through cheesecloth, and stored in the cold room after being saturated with chloroform.

(2) A preparation made according to the method of Gladstone and Fildes (1940) has proved satisfactory. It is more convenient to use one of the commercial powders now available, and our experiments have been carried out for the most part with two products of the Sheffield Farms Company—"pepticase" and "N-Z-case." It should be noted that one batch of the former was completely useless, for reasons which we could not determine, although all others tested have given uniformly good results.

(3) Added from a 1 per cent stock solution in 2 per cent HCl.

(4) As in (3) and required only if tyrosine has largely crystallized out of the digest and been discarded.

(5) Stock solution:

Na_2HPO_4..	2.0 g
KH_2PO_4..	0.7 g
$MgSO_4 \cdot 7H_2O$..	0.2 g
Water to 100 ml	

To the ingredients of the negative control contained in a series of 6-by-$\frac{3}{4}$-inch tubes with pointed ends (for centrifuging) are added those substances or fractions which are to be tested as replacements for the whole tryptic digest. The pH is adjusted to 7.0 to 7.2 with HCl or NaOH, the volumes are brought to 20 ml with water, and 5 to 10 mg of reduced iron powder (Merck) are added to each tube. After being covered with glass caps, the tubes are sterilized for 20 minutes in flowing steam (100 C) and cooled in water, the glass caps are replaced with sterile cotton plugs, and the tubes are inoculated with 0.1 to 0.2 ml of a 24-hour broth culture of *C. tetani* prepared as described elsewhere (Mueller and Miller, 1945). Incubation is at 35 C, best ± not over 0.1 degree, obtainable in a well-stirred water bath with a sensitive thermostat. On the fifth day the tubes are centrifuged for 5 minutes, the supernatants, which need not be entirely clear, are decanted into clean tubes in order to ensure thorough mixing, and flocculations are carried out at 42 C over a range of 10 to 120 Lf per ml. Failure to obtain flocculation in 1 hour at a level of 10 Lf is recorded as "nil," although a titer of 10 Lf (= 100 test doses or approximately 100,000 mouse MLD) is about the level at which toxin was obtained in our earlier work on "peptone-free" media, and can pass National Institute of Health minimum requirements. The positive control at a level of 100 to 110 Lf should flocculate in 5 to 7 minutes.

A titer of intermediate value between "nil" and maximum cannot as yet be definitely interpreted since, as will appear, it is now quite clear that at least two factors take part in the growth and formation of toxin. There is a strong suggestion that both of these are, in turn, multiple. Until each of the substances can be omitted from a base containing all the others, it will be impossible to know whether its absence prevents or simply diminishes the formation of toxin. Obviously, the absence of the tryptic digest must cause a relatively complex multiple deficiency for growth, since there remains no source of amino acids save minimal amounts present in the beef heart infusion. The requirements for growth on the part of our strain have been determined and include a dozen or more amino acids. Unfortunately, the attempted inclusion of these amino acids in the base has not been practicable, since some of them seem to inhibit the formation of toxin. It has been necessary at all times to keep these facts in mind and to attempt to avoid confusing an accidental deficiency of one or more amino acids with the absence of a more complex and labile grouping, and at the same time to interpret intermediate titers conservatively in respect to the particular point under investigation.

The foregoing considerations have resulted in an inclination to regard maximal yields as the objective of each experiment. These are never obtained except when all conditions are fulfilled. A titer of 30 to 50 Lf may be due to one or more of a variety of reasons and must, for the present, be interpreted tentatively.

It may be worth while to describe the appearance of a satisfactory culture as it develops. Ten to 20 hours after inoculation growth becomes evident deep in the medium and rapidly extends throughout. In 18 to 20 hours it is fairly heavy; moderate evolution of gas is taking place, and the contents of the tube are dark in color presumably because of the formation of colloidal FeS. During the second day turbidity, gas formation, and darkening increase progressively.

Sometime during the third day the FeS begins to separate as a flocculent precipitate and by the fourth day has settled out, leaving a relatively colorless supernatant in which the bacteria appear to have undergone autolysis. Gas production has diminished. By the fifth day the whole process has stopped, and no further change occurs if incubated longer. The final pH is 7.2 to 7.4. Flocculations carried out at intervals during incubation show little or no toxin until the FeS begins to settle and autolysis commences. The possibility that tetanus toxin is formed intracellularly and liberated only on disruption of the cell has been pointed out by Bayne-Jones (1945), and our observations tend to confirm such a view.

Cultures on media deficient in one respect or another behave somewhat differently. Frequently they become blacker than the control, the dark color persists, and even after 5 days the FeS has not settled. Occasionally, on the other hand, clearing occurs even before the control. The degree of opacity due to bacterial growth is difficult to assess because of the dark color. Careful observation will usually detect a greater degree of turbidity in those tubes which later prove to have formed good toxin. One point which may merit particular mention is that autolysis may fail to occur in tubes in which growth is moderately good, and under this circumstance toxin is usually not found. Whether one or several types of deficiency are responsible for this condition is not yet clear, and attempts to induce autolysis by modification of pH, etc., in these tubes have thus far been unsuccessful.

It would be unprofitable to attempt a complete presentation of experiments directed at a separation of active material from the tryptic digest. Such experiments now extend well over 3 years, and the majority are indecisive or negative. A selection will be made of a few to illustrate conclusions which one may be justified in drawing with reasonable assurance at this stage of the work.

Source of toxigenic material. Commercial casein is in many respects a poorly defined protein and is possibly an unfortunate choice as a starting material for an extended program of investigation. In its favor are low cost and uniformity within a single large batch. It seemed important, however, to determine if possible whether the substances which were sought originated in the protein molecule, were present as impurities in the casein, or had their origin in the enzyme preparation employed for the digestion.

Tryptic digests were consequently prepared from 10 grams each of (a) bovine albumin,[2] (b) bovine hemoglobin, (c) commercial casein, and (d) vitamin-free casein (Harris), following the procedure given by Gladstone and Fildes (1940) except that 0.3 g pancreatin powder (Merck) were used in place of minced hog pancreas. At the end of 15 days digestion media were prepared using these four products in place of the usual tryptic digest. The results are shown in table 1.

Since an acceptable, though not maximal, amount of toxin was formed regardless of the type of protein, the conclusion seems warranted that an important fraction of the total effect is due to fragments of protein, rather than to extra-

neous materials.   A possible contributory effect of the enzyme preparation cannot be excluded at this stage of the work, but of itself, and in the presence of a moderately complete assortment of amino acids, it is without effect.   The failure to obtain maximal yields is in part attributable to failure to adjust carefully the quantities used as to nitrogen content, etc.   This would have proved time-consuming and could not have altered the conclusions significantly.

Failure of acid hydrolyzate of casein to replace tryptic digest.   The experiment summarized in table 2 demonstrates that a fully supplemented acid hydrolyzate does not yield a medium suitable for the production of a potent toxin.

Solubility of essential factors.   As might be anticipated, the addition of ethyl alcohol to the tryptic digest in sufficient quantity brings down considerable

TABLE 1

Production of toxin on media containing digests of various proteins

TUBE NO.	COMPOSITION	TOXIN TITER IN 5 DAYS
		Lf/ml
1	Base + N-Z-case 300 mg	80
2	" + tryptic digest of bovine albumin	40
3	" " " bovine hemoglobin	25
4	" " " commercial casein	40
5	" " " "vitamin-free" casein	40

TABLE 2

Substitution of acid hydrolyzate of casein for tryptic digest

TUBE NO.	COMPOSITION			TOXIN TITER IN 5 DAYS
				Lf/ml
1	Base + tryptic digest 300 mg + HCl hydrolyzate	0		90
2	" " " 225 "	" "	75 mg	75
3	" " " 150 "	" "	150 "	43
4	" " " 75 "	" "	225 "	20
5	" " " 0 "	" "	300 "	10

insoluble, amorphous, and "oily" material, which carries activity with it, but there is no evidence of separation of two components or of significant concentration.

Prolonged extraction of a neutral solution of tryptic digest with butyl alcohol separates a certain amount of inactive material and therefore achieves a measure of concentration of the material in the residue, but not to a degree which appears to warrant the use of such a method as a practicable initial step in devising a method for isolation.   Extraction with this solvent from either acid or alkaline solution has failed to supply any promising lead.   Less polar solvents of course fail to remove appreciable amounts of solid, and do not influence activity.   These results do not warrant illustration by specific protocols.   The use of phenol as a solvent will be considered later.

Adsorption. Extensive attempts have been made to remove selectively some active component of the tryptic digest by means of adsorption on charcoal, and to recover it by elution. Norit or "nuchar 190-N" will remove activity from either neutral or slightly acid solutions, but the adsorption does not seem to be specific, and a considerable amount of the adsorbent must be used before activity is completely lost. By color tests it has been shown, for example, that tryptophan and tyrosine are readily removed with moderate amounts of charcoal. Provided the deficiency is rectified with pure amino acids, filtrates so obtained are satisfactory for the production of reasonably good, although not maximal, toxin. Eluates of the charcoal with dilute alcohol or alcoholic ammonia have behaved irregularly in the degree to which they will reactivate the medium. Attempts to employ charcoal columns chromatographically have failed to show promise of success in the fractionation of the crude hydrolyzate, although the possibility exists that after suitable preliminary procedures, the method might have considerable value.

Similar conclusions have been reached in regard to the use of the reversible resins for the separation of activity. Amberlites IR-4 and IR-100 and "zeokarb" are all capable of progressive removal of activity and of yielding a portion only of active material when eluted with appropriate electrolytes.

As a result of prolonged experimentation with these various adsorbents, the conviction grew that the essential material in the tryptic digest was multiple. In no single experiment, however, was it possible to separate sharply two fractions, each without activity, yet together producing a normal yield of toxin. It was therefore uncertain whether actual separation was accomplished by any particular step, or only that one or more substances were simply spread throughout all fractions. In spite of this absence of positive proof, it became increasingly probable that at least two, and possibly several, components of the digest were involved.

Resistance of active material to acid and alkali. Knowledge of the fact that hydrolysis by strong acid destroyed activity of the tryptic digest had influenced us to examine first the available methods of separation not requiring prolonged exposure to reactions far from the neutral point. Repeated failure indicated the necessity of exploring somewhat more drastic methods. As a preliminary measure experiments were conducted to determine the maximal amounts of acid and alkali to which the crude active material was stable.[3] It was of course realized that, if two or more factors were concerned, one might be considerably less resistant than another, and the most labile substance would limit initially the choice of methods for separation.

It was found that toxin of good, though of gradually diminishing, titer could be produced after 1 to 4 hours' heating of 10 per cent solutions with 2 N acid and alkali, although eventually total loss of toxigenicity resulted.

A single experiment may serve to illustrate the type of result obtained (table 3). Ten grams of N-Z-case were dissolved in 100 ml 2 N H_2SO_4. Ten ml of the

resulting solution were immediately removed, and the acid was neutralized with Ba(OH)₂. This sample served as a control of any immediate effect and of possible loss on the BaSO₄ precipitate. The remaining solution was boiled under a reflux condenser, 10-ml samples were taken as indicated, the H₂SO₄ was removed, and the products were tested for ability to induce toxin formation after they were added to the usual basic control medium.

From this and similar experiments conducted by heating with either H₂SO₄ or Ba(OH)₂ solutions it became evident that the substances leading to toxin formation were not immediately destroyed by either acid or alkali, and that in consequence a reasonable amount of chemical manipulation could be contemplated.

Chemical precipitation Ba(OH)₂ and alcohol, followed by silver. As a result of a number of small-scale experiments, a procedure was chosen following in a general way that for the fractionation of the amino acids in a complete acid hydrolyzate of protein. Briefly, hot Ba(OH)₂ was added in moderate excess to

TABLE 3

Effect of boiling with 2 N H₂SO₄ on toxigenic factors

TUBE NO.	MATERIAL ADDED TO CONTROL BASE	TITER IN 5 DAYS
		Lf/ml
1	Tryptic digest of casein, untreated	110
2	Same dissolved in 2 N H₂SO₄ and acid immediately removed	100
3	" refluxed 5 minutes and acid removed	90
4	" " 30 " " " "	70
5	" " 1 hour " " "	60
6	" " 2 hours " " "	55
7	" " 4 " " " "	35
8	" " 24 " " " "	5(?)

a solution of the tryptic digest followed by three volumes of 96 per cent alcohol. The *precipitate* was removed, freed from Ba with H₂SO₄, and concentrated somewhat *in vacuo* to eliminate remaining alcohol. The Ba-alcohol *filtrate* was neutralized with H₂SO₄, concentrated *in vacuo* to remove alcohol, and freed from excess Ba or SO₄. At this stage there was evidence of participation of both fractions in toxin formation. The barium precipitate, with little or no activity when used alone, appeared to increase the moderate activity of the filtrate fraction, lending support to the view that at least two substances were involved.

The barium *filtrate* was further subdivided by precipitation with silver. Ag₂O and H₂SO₄ were added alternately until an excess of silver was present. A small amount of precipitate separating in acid reaction was removed. Ba(OH)₂ was added to the filtrate and a voluminous precipitate separated between pH 7 and pH 8. This was removed and further additions of Ba(OH)₂ were made until the pH rose to 10 or above. The two precipitates were centrifuged separately, and these together with the final supernatant were freed from Ba with H₂SO₄, and from Ag with H₂S.

Phosphotungstic acid precipitation of the Ag supernatant fraction was then accomplished after acidifying with H_2SO_4 to about pH 3. The pH of course fell much lower as a result of the reagent (20 per cent phosphotungstic acid in 10 per cent H_2SO_4). The precipitation was carried out in an ice-cooled bath, after which the precipitate was promptly centrifuged in a refrigerated centrifuge. The excess reagent was removed as quickly as possible with $Ba(OH)_2$, and the solutions were restored to neutrality with dilute H_2SO_4.

Schematically the fractions obtained were as diagramed in figure 1.
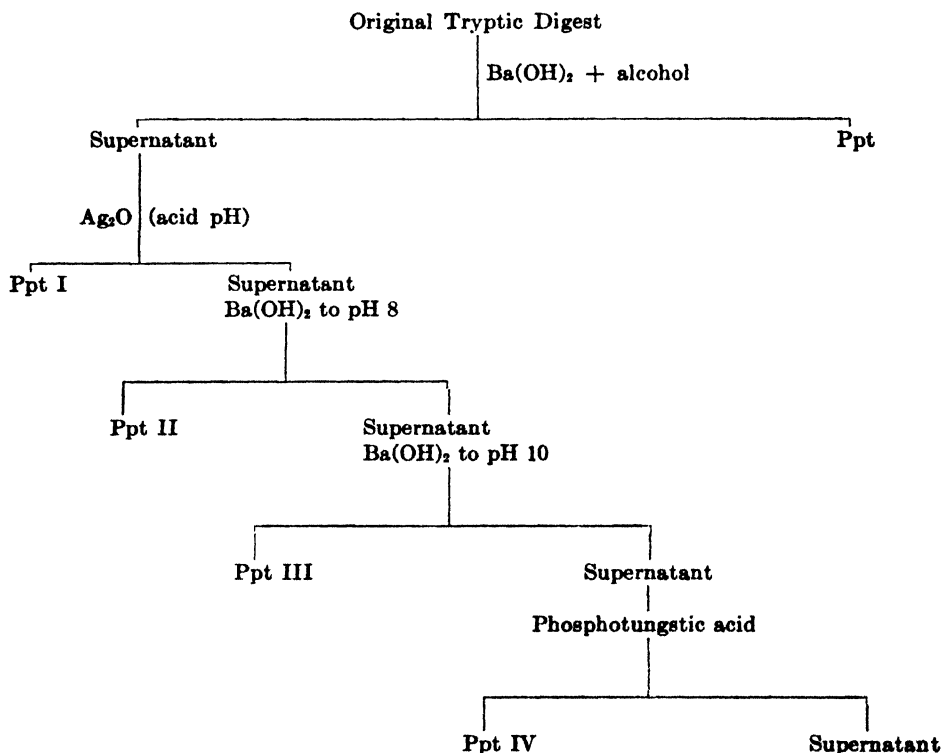
Figure 1

The acid silver precipitate and the final phosphotungstic supernatant were without demonstrable activity. All the other fractions appeared to contribute in some degree to the formation of toxin of medium titer. Full activity was never recovered, and it remained uncertain whether this was due to partial inactivation or to cumulative losses on the abundant inert precipitates which separated in the various steps. Various attempts at chemical separation of an active component from any of these main fractions failed. Invariably with each step there was loss, until the difference between controls and tests was no longer sufficiently definite to assure validity of observation.

The sum total of attempts at direct chemical fractionation have therefore been

discouraging, although the possibility remains that certain portions of the information which has emerged may be successfully applied at a later stage of the work.

Countercurrent extraction. The great possibilities for separation of mixtures offered by the "counter-current" extraction method of Craig (1944), provided a suitable solvent is available, led to an attempt to explore by his technique the use of certain strongly polar organic substances. Guided by the fact that both phenol and collidine (the latter only at temperatures of 60 C or below in our hands) are much more effective than butyl alcohol in the separation of amino acids on the paper strip chromatograms of Consden, Gordon, and Martin (1944), preliminary small-scale experiments with the former and with lutidine were performed. Lutidine was selected rather than collidine since it dissolves more water at room temperature than does the latter. Both solvents showed considerable promise, and phenol in particular seemed worthy of intensive investigation.

Phenol at room temperature takes up about 15 per cent of water to form a heavy oil. Water, on the other hand, dissolves somewhat more than 10 per cent of phenol. When shaken together, therefore, equal parts of these substances separate sharply into two layers, each saturated with respect to the other.

A 10 per cent solution of tryptic digest of casein was prepared and centrifuged free of undissolved tyrosine. It was then saturated with phenol crystals. The solution remained essentially clear. Ten ml of this solution were thoroughly shaken in a test tube with 10 ml of phenol saturated with water. After standing for a short time the two layers separated sharply. The phenol layer was pipetted into a second tube containing 10 ml of a saturated aqueous solution of phenol, and 10 ml more of liquid phenol were added to the first tube. Both tubes were shaken, and, after separating, the phenol of the second tube was transferred to a third, that from the first to the second, fresh phenol was added to the first, and fresh water to the third. By a continuation of this process the extraction was carried through six tubes ("plates"). It was evident that a very appreciable amount of the original solids was being carried along by the phenol. Emulsions tended to form in some of the tubes, which, however, were easily broken by brief centrifuging. To determine whether activity could now be recovered, or whether loss had resulted through some effect of the phenol, 3 ml of each phenol layer and of each water layer were combined and the phenol was removed by extracting it several times with ether. The aqueous layer was finally concentrated to 10 ml by vacuum distillation. Addition of this solution to the basic medium produced an essentially maximum titer of toxin.

The experiment was then carried out on a larger scale, employing 250-ml separatory funnels rather than test tubes, making it possible to start with 100 ml of a solution of tryptic digest and 100 ml of phenol. This experiment was carried through twelve "plates." Aliquots of the final twelve mixtures were removed, combined into three fractions: (A) plates 1 to 4, (B) plates 5 to 8, and (C) plates 9 to 12, and each freed from phenol with ether. Substitution of these fractions in the basic medium gave the results indicated in table 4.

This experiment, which has been repeatedly confirmed, represents the first

successful separation of two components (or groups), each essentially without activity of itself but together giving practically the complete effect of the original. It seems probable that fraction B contains low concentrations of the components of both A and C, and therefore alone is capable of some toxin production. Fractions A and C, on the other hand, being relatively well separated from each other, are individually almost without effect, while together they form a nearly complete medium.

A more extensive separation of a larger amount was then undertaken. A 30 per cent solution of tryptic digest was used which could be obtained (except for tyrosine) by warming. It was allowed to cool and to stand for some hours, during which time a moderate amount of granular, semicrystalline material (leucine?) separated. This was discarded without loss of toxigenicity. One hundred ml of the rather heavy, syrupy solution so obtained was shaken with 100 ml of phenol. The resulting emulsion separated slowly, but separation could be expedited by transferring the mixture to a 250-ml cup and centrifuging

TABLE 4

Separation of toxigenic factors by phenol-water partition

TUBE NO.	MATERIAL ADDED TO BASE	TOXIN IN 5 DAYS
		Lf/ml
1	N–Z-case 300 mg	95
2	Fraction A (equivalent to 300 mg N–Z case)	nil
3	" B (equivalent to 300 mg N–Z-case)	30
4	" C (equivalent to 300 mg N–Z-case)	nil
5	" A + B (equivalent to 300 mg N–Z-case)	17
6	" A + C (equivalent to 300 mg N–Z-case)	70
7	" B + C (equivalent to 300 mg N–Z-case)	nil
8	" A + B + C (equivalent to 300 mg N–Z-case)	80

briefly. At this stage the aqueous solution was heavier than the phenol layer. After separating the two layers, progressive extractions were carried forward until 24 plates had been separated. After the third extraction, the original aqueous layer became lighter than phenol, and from this point the separation proceeded normally. The method was laborious but not prohibitively so. About 2 days were required to complete the 24 extractions.[4] Emulsions which were progressively difficult to break occurred after the first few plates, about two-thirds of the way down the row of separatory funnels. Whatever the substance responsible for this phenomenon, it traveled progressively to the right as the extraction proceeded and necessitated the centrifuging at first of a single fraction, later of three or four adjoining ones.

Aliquots of these 24 plates have been freed from phenol and tested in various combinations. The essential facts of the earlier 12-plate experiment have been

[4] The extractions were greatly facilitated by mixing the contents of the funnels at a time, on a rotating support constructed for the purpose by Mr. Sam Levin department (Proc. Soc. Exptl. Biol. Med., *in press*).

confirmed, and in addition certain of the results indicate that each of the two well-separated fractions may be further resolved into at least two more components.

At this point an attempt was made to integrate portions of the results of earlier experiments with the information made available by phenol countercurrent extractions, and the following observations were made: (1) Substitution of silver precipitate fractions for various groups of plates in the 24-plate experiment, in the presence of a base containing the material from all remaining plates, indicates that the silver-precipitable material replaces reasonably well the substances in plates 13 to 20. Possibly it is significant that the constituents of these plates, as well as of the silver precipitates, include a good deal of histidine-containing peptide. (2) It appears that if a 10 per cent solution of tryptic digest is treated with nuchar 190-N in sufficient quantity (15 per cent) to render it inert as far as toxin production is concerned, activity is largely restored both by plates 13 to 20 and by silver precipitate.

This last observation seems to offer a reasonable plan for a direct attempt at the isolation of a single one of the multiple factors involved in toxin formation. If the charcoal-treated digest is indeed completely deficient in one factor, although perhaps diminished in concentration with respect to one or more others, and if this factor is present in plates 13–20 as well as in silver precipitate, a practicable basic medium for its assay is readily available. This possibility is being investigated as described in the following section.

Starch column chromatography. Paper strip chromatograms of the material in plates 13 to 20 as well as in the silver precipitate show that each still represents a relatively complex mixture. The fact that a considerable range of mobility is manifested in all solvents used (butanol, phenol, collidine, lutidine) strongly suggests that a suitable chromatographic method of separation might offer the greatest promise of success. Moore and Stein (1948) have shown that it is possible to use potato starch columns for the separation of amino acids and of the simpler peptides. Their work represents an extension and refinement of earlier partially effective attempts in the same direction by Synge (1944). From mathematical considerations these workers calculate that a starch column 30 cm in length is capable of a degree of resolution of the components of a mixture corresponding to several thousand theoretical plates. A number of preliminary experiments were carried out according to a procedure many of the details of which were generously supplied to us by Drs. Moore and Stein. The following illustrates one in which relatively complete and clear-cut results were obtained.

A filter-tube 15 by 2 cm was lightly plugged at the bottom with glass wool, and this in turn was covered with a thin, compact layer of filter paper pulp. A suspension of 100 g of potato starch in 150 ml butanol and 17 ml water was poured into the tube, and air pressure of about 5 cm of mercury applied. Further additions of starch suspension were made from time to time until a column about 12 cm long was obtained. This was washed with about 50 ml of 90 per cent butanol. Two ml of a 10 per cent solution of 8-hydroxyquinoline in 90

per cent butanol were then run on the column, and the washing was continued with another 50 ml of the same solvent.

The phenol was removed from portions of the combined plates 13 to 20 equivalent to 1.2 g of the original tryptic digest and containing about 160 mg of solids, the solution was concentrated in an air current almost to dryness and taken up in 0.4 ml of 2.5 N HCl. This was mixed with 5 ml of anhydrous butanol and put on the column. Development was with 90 per cent butanol and was continued until 13 serial fractions of filtrate of about 9 ml each had been collected. A loopful of each fraction was dried on filter paper, sprayed with ninhydrin, and warmed. Tubes 1 to 4 showed no reaction, whereas a very strong positive reaction was shown in tubes 5, 6, and 7, little in 8, somewhat more in 9, and then it gradually diminished to doubtful tests in 12 and 13.

The starch column itself was then carefully removed from the filter tube, and samples of a few mg of starch were taken at various levels and tested with ninhydrin. A weekly positive reaction was obtained at the lower end of the tube, and a strongly positive one at the top. The main bulk of the starch was then divided in the middle, and each half was extracted repeatedly with small amounts of water.

All fractions were freed from butanol by concentration *in vacuo*, and portions corresponding to 40 mg of original solid were tested for toxin production in the usual basic medium without N-Z-case but containing the charcoal filtrate referred to above together with tryptophan.

It is evident that the most active of the fractions is the one that shows little or no tendency to pass down the starch column when 90 per cent butanol is used as the solvent. This conforms to what could have been anticipated from earlier experiments, to which allusion has already been made, which showed little or no tendency for activity to pass from water to butanol. The moderate increase in activity when all fractions were mixed (table 5, tube no. 9) has been confirmed in a number of similar subsequent experiments, using both phenol plates 13 to 20 and silver precipitate as starting material. The effect was found to be due to something in the effluent from the column and could be largely if not entirely replaced by phenylalanine. The presence of phenylalanine in the effluent can be demonstrated by paper chromatograms and colorimetric tests.

Phenylalanine is one of the amino acids known to be required or stimulatory in the growth of our organism and is the first of these, with the exception of cystine, tyrosine, and tryptophan, to be shown to be effective in the free form in toxin production under these conditions. The effect is demonstrated in the following experiment (table 6):

Silver precipitate material nearly 2 years old was used. Instead of passing the entire preparation through a starch column, a separation of a very concentrated solution of the hydrochlorides was brought about by shaking with 10 volumes of anhydrous butyl alcohol and centrifuging. The oily "residue" had been shown in earlier experiments to replace the "top of the column" fraction of the experiment detailed above.

Stein and Moore have shown that the basic amino acids, which show little

mobility on starch with 90 per cent butanol, can be separated if mixtures of propyl alcohol and aqueous hydrochloric acid are used for the development. The possibility of loss in activity through prolonged exposure of the "butyl residue" material to acid led us to experiment briefly with starch columns in which either 85 per cent phenol or water-saturated lutidine were employed as solvents. Both types of column gave encouraging results, but each presented certain difficulties in operation. Before attempting to perfect either one, a small column was tried using propanol and aqueous 0.5 N H_2SO_4 in the proportion

TABLE 5

Effect of fractions obtained on starch column with butanol

TUBE NO.	MATERIAL ADDED TO BASE		TOXIN IN 5 DAYS
			Lf/ml
1	N–Z-case 300 mg		100
2	Charcoal filtrate + tryptophan + tyrosine		10
3	" " " "	+ plates 13–20	75
4	" " " "	+ tubes 1–4	nil
5	" " " "	" 5–7	10
6	" " " "	" 8–13	nil
7	" " " "	bottom of column	10
8	" " " "	top " "	55
9	" " " "	all 5 fractions	65

TABLE 6

Effect of phenylalanine on toxin formation

TUBE NO.	MATERIAL ADDED TO BASE		TOXIN IN 5 DAYS
			Lf/ml
1	N–Z-case 300 mg		105
2	Charcoal filtrate + tryptophan + tyrosine		15
3	" " " "	+ butyl residue	15
4	" " " "	" " "	25
	+ DL-phenylalanine 0.1 mg		
5	Charcoal filtrate + tryptophan + tyrosine + butyl residue		70
	+ DL-phenylalanine 1.0 mg		
6	Charcoal filtrate + tryptophan + tyrosine + butyl residue		60
	+ DL-phenylalanine 10.0 mg		

of 2:1. The fractions of filtrate so obtained were neutralized with barium hydroxide, the $BaSO_4$ was centrifuged out, and the propanol was removed by vacuum distillation. It has been possible to demonstrate activity in the filtrate, and also to show by paper chromatograms that a fair degree of separation of the several components present in the "butyl residue" is achieved.

Preparations are now under way for the separation of a sufficient quantity of the active material in the silver precipitate for chemical examination. The writers hope to report more definitely on its nature, as well as on the results of

applying starch chromatography to the other factors already partially separated by phenol countercurrent extraction.

CONCLUSIONS

A tryptic digest of commercial casein contains at least two substances destroyed in ordinary acid or alkaline hydrolysis that appear essential for the production of potent tetanus toxin under the conditions of these experiments.

Fairly strong toxin has been obtained from tryptic digests of "vitamin-free" casein and of bovine albumin and hemoglobin, indicating the probability of a widespread distribution of the compounds or groupings involved. The possibility that the trypsin preparation contributes in some way to the effect has not yet been excluded.

The most promising procedure for the isolation of the substances which are concerned appears at present to be an initial separation by "counter-current" extraction with phenol, followed by chromatographic fractionation on starch columns.

By this means phenylalanine has been identified as being concerned in toxin formation, and a concentrate, active at a level of 0.2 to 0.4 mg in 20 ml of medium, has been obtained, but not as yet in a sufficiently pure condition for chemical characterization. Further investigation of this material, as well as of the one or more additional unknown components of the basic medium, is in progress.

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THE EFFECT OF "TWEEN 80," BOVINE ALBUMIN, GLYCEROL, AND GLUCOSE ON THE GROWTH OF MYCOBACTERIUM TUBERCULOSIS VAR. HOMINIS (H37Rv)[1]

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In a series of publications (Dubos, 1945; Dubos and Davis, 1946; Davis and Dubos, 1946a; Davis and Dubos, 1946b; Dubos, 1946; Dubos, Davis, Middlebrook, and Pierce, 1946; Dubos, 1947; Davis and Dubos, 1947; Dubos and Middlebrook, 1947) the addition of certain wetting agents to synthetic culture media used for the growth of virulent tubercle bacilli has been advocated. The synthetic nonionic detergent "tween 80" was found to be the most suitable. The advantages claimed were that this substance exerted a stimulating effect on the growth of tubercle bacilli and produced a diffuse homogeneous growth similar to that of many non-acid-fast bacteria instead of the granular or flaky growth usually encountered. Commercial preparations of tween 80, however, contained a sufficient amount of unesterified oleic acid to exert a growth-inhibitory effect on small inocula of tubercle bacilli. This could be removed chemically from the commercial material or counteracted by the addition of purified serum albumin to the medium. These investigators also reported that glucose and glycerol increased the total amount of growth of tubercle bacilli but not the initial rate of multiplication, and that glycerol exerted an inhibitory effect on the growth of small inocula in the presence of tween 80 that was not apparent in the absence of the oleic acid ester.

Quantitative determination of the growth rate of *Mycobacterium tuberculosis* var. *hominis* has been difficult because of the lack of reliable methods. Youmans (1946), however, has shown that nitrogen determinations can be used for accurately determining the amount of growth of tubercle bacilli, and the effect that alteration of the composition of the medium and the presence of growth inhibitory substances has on the rate of growth. In this manner the effect of various substances on the rate of growth may be evaluated on a quantitative basis. This technique is necessitated by the fact that, without a dispersing agent in the medium, tubercle bacilli form flaky clumps, which eliminates the application of turbidimetric, plating, or direct counting methods for the estimation of the amount of growth.

METHODS

Basal media. Two chemically defined media were employed. The first was the modified Proskauer and Beck synthetic medium (modified P & B synthetic medium), which has previously been employed by Youmans (1946). The

second medium was that recommended by Dubos (1947), in which we employed asparagine as the nitrogen source. Only water redistilled from glass was employed. The pH of both media was adjusted to 7.0 using a Macbeth electrometer, and the media were sterilized in the autoclave at 10 pounds for 20 minutes.

Tween 80 (polyoxyethylene sorbitan monooleate), a synthetic nonionic detergent, was obtained from the Atlas Products Company of Wilmington, Delaware. Three fresh lots (ARL no. 3020, no. 3292, and no. 7939) of tween 80 were employed, and were stored in the icebox at 5 C. The purified oleic acid-free tween 80 was prepared from one of these (Lot no. 3020) as required, according to the method recommended by Davis (1947).[2] With this lot 0.60 per cent acid, calculated as oleic acid, was extracted from the unpurified tween 80 and the yield of purified tween 80 was approximately 70 per cent. These results compare very favorably with those obtained by Davis (1947) in the purification of tween 80. Tween 80 was employed in the media in a concentration of 0.05 per cent. All solutions of tween 80 were freshly prepared just before being used.

Albumin. Bovine albumin powder (fraction V) prepared by Armour Laboratories, Chicago, Illinois, was employed. A 5.0 per cent solution in physiological saline was inactivated by heating the albumin solution for 30 minutes at 55 C to remove any substances that might liberate free oleic acid by enzymatic action after prolonged incubation with the water-soluble ester tween 80 (Dubos and Davis, 1946). Sterile solutions were then obtained by filtration through porcelain candles (Selas no. 2) or Berkefeld filters (normal porosity) and added to the autoclaved medium with aseptic precautions in amounts to give a final concentration of 0.2 per cent.

Glassware. Pyrex test tubes, 200 by 25 mm, were cleaned by standing them overnight in concentrated sulfuric acid or detergent cleaning solution.[3] They were rinsed then 7 to 8 times with running tap water, 3 times with distilled water, and twice with redistilled water. When dry they were capped with loose-fitting aluminum caps and sterilized in the autoclave at 20 pounds pressure for 20 minutes. Following sterilization, 10.0 ml of sterile medium were introduced aseptically into each tube with an accurately calibrated volumetric pipette.

Determinations of growth of tubercle bacilli. The method employed for the determination of the amount of growth of tubercle bacilli in the various media by micro-Kjeldahl nitrogen determinations followed the procedure used by Youmans (1946).

The virulent human type strain, H37Rv, of *M. tuberculosis* was employed in all experiments. The amount of inoculum used ranged from 0.042 to 0.070 mg bacterial nitrogen per 10.0 ml of medium, but the inoculum was constant in all experiments in which comparisons were being made.

RESULTS

The effect of unpurified tween 80 on the growth of H37Rv in modified Proskauer and Beck synthetic medium. Graph 1 shows a typical experiment in which 0.05 per cent unpurified tween 80 was added to the medium. It is apparent that the tween 80 exerted an inhibitory action on the growth of the tubercle bacilli. The actual decrease in the rate of growth in the presence of tween 80 as determined from the straight-line portions of the curves was 37.1 per cent. Table 1 shows the results of 7 similar experiments in which the percentage of inhibition of growth rate produced by 0.05 per cent tween 80 varied from 28.3 to 49.8.

TABLE 1

Effect of unpurified tween 80 (0.05 per cent) on growth of tubercle bacilli (H37Rv) in modified P & B synthetic medium

EXP. NO.	CONCENTRATION OF TWEEN 80 IN PER CENT	MILLIGRAMS BACTERIAL NITROGEN						PER CENT OF INHIBITION OF GROWTH RATE
		Inoculum	Time in hours					
			68	116	164	212	260	
A	0.00	0.059	0.119	0.177	0.289	0.388		
	0.05	0.059	0.088	0.124	0.150	0.227		37.7
B	0.00	0.070	0.117	0.179	0.263	0.335	0.507	
	0.05	0.070	0.084	0.125	0.173	0.195	0.267	38.2
C	0.00	0.042	0.102	0.176	0.246	0.313	0.431	
	0.05	0.042	0.064	0.083	0.118	0.132	0.158	49.8
D	0.00	0.053	0.106	0.150	0.189	0.249	0.313	
	0.05	0.053	0.079	0.112	0.134	0.168	0.204	28.3
E	0.00	0.049	0.126	0.202				
	0.05	0.049	0 072	0.108				44.2
F	0.00	0.047	0.119	0 211				
	0.05	0.047	0.078	0.129				32.8
			72 hr	120 hr	168 hr			
G	0.00	0.049	0.109	0.161	0.245			
	0.05	0.049	0.071	0.103	0.140			37.1

0.01 mg bacterial nitrogen is equivalent to approximately 0.23 mg of dried tubercle bacilli.

The effect of purified tween 80 on the growth of H37Rv in modified Proskauer and Beck synthetic medium. Graph 1 also shows the effect of purified tween 80 on the growth of the H37Rv strain. This purified material inhibited growth 23.3 per cent. Also shown in the graph is the effect on the growth of tubercle bacilli of the unpurified tween 80 from which the purified material was prepared. This produced considerably more inhibition of growth than the purified material. Two similar experiments with other preparations of purified tween 80 produced 20.7 and 24.9 per cent inhibition of growth, respectively.

The effect of purified and unpurified tween 80 on the growth of H37Rv in Dubos basal medium. Similar experiments were conducted using Dubos basal medium,

which contained glucose instead of glycerol, and in every case the same inhibitory effect of both purified and unpurified tween 80 was observed.   The degree of inhibition by these agents was usually slightly less than with the Proskauer and Beck modified synthetic medium.   However, accurate determinations of the degree of inhibition were difficult to measure since the rate of growth in this medium fell off so much more rapidly than in the modified Proskauer and Beck
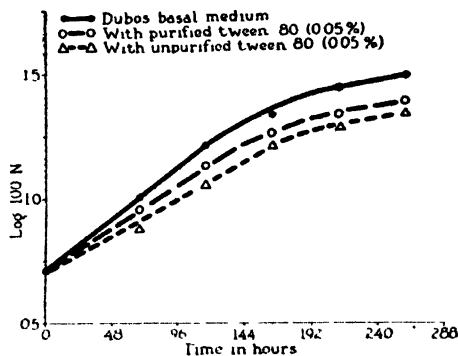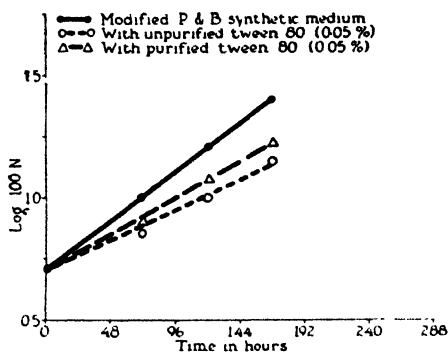
Graph 1, upper left. Comparison of the effect of purified and unpurified tween 80 (0.05 per cent) on growth of *M. tuberculosis* (H37Rv) in modified P and B synthetic medium.
Graph 2, upper right.   Comparison of the effect of purified and unpurified tween 80 (0.05 per cent) on growth of *M. tuberculosis* (H37Rv) in Dubos synthetic basal medium.
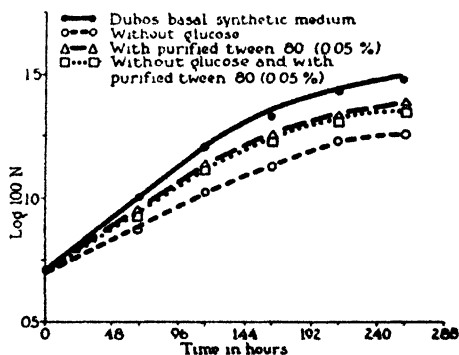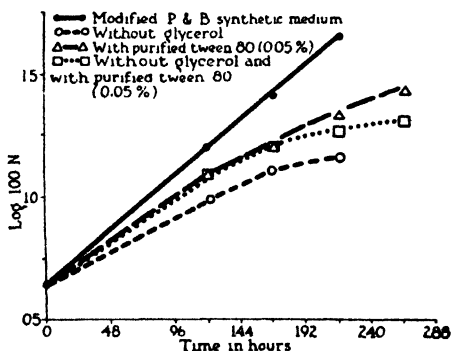
Graph 3, lower left. Effect of glycerol (2.0 per cent) and purified tween 80 (0.05 per cent) on the growth of *M. tuberculosis* (H37Rv) in modified P and B synthetic medium.
Graph 4, lower right.   Effect of glucose (0.2 per cent) and purified tween 80 (0.05 per cent) on growth of *M. tuberculosis* (H37Rv) in Dubos basal synthetic medium.

medium that it was difficult to find straight-line portions of the curve to use for this purpose.   Graph 2 shows a typical experiment in which the unpurified tween 80 inhibited growth approximately 30 per cent, and the purified tween inhibited growth approximately 13 per cent, using the rate of growth during the first 5 days as the basis for the comparison.

Effect of glycerol[4] *(2.0 per cent) and glucose (0.2 per cent) and purified tween 80*

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[4] Baker and Adamson reagent grade glycerin.

(0.05 per cent) on the growth of M. tuberculosis (H37Rv) in modified Proskauer and Beck and Dubos basal synthetic medium. Graph 3 shows the results obtained with modified Proskauer and Beck medium containing (1) 2.0 per cent glycerol; (2) 2.0 per cent glycerol plus 0.05 per cent tween 80; (3) 0.05 per cent tween 80; (4) neither glycerol nor tween 80. It is evident that omission of the glycerol resulted in marked reduction in the rate of growth of the tubercle bacilli (37.6 per cent). The addition of 0.05 per cent tween 80 to this medium containing no glycerol resulted in a stimulation of growth but not to the same degree as the addition of glycerol. The addition of both glycerol and tween 80 to the basal medium resulted in a rate of growth for a period of 7 days that was the same

TABLE 2

Effect of albumin (0.2 per cent) and tween 80 (0.05 per cent) on growth of tubercle bacilli (H37Rv) in modified P & B and Dubos media

EXP NO.	TYPE OF MEDIUM	ALBUMIN	PURIFIED TWEEN 80	UNPURIFIED TWEEN 80	MILLIGRAMS BACTERIAL NITROGEN					
					Inoculum	Time in hours				
						72	120	168	216	264
H	Modified P & B	—	—	—	0 046	—	0.132	0.210	0 288	0.372
	"	0.2%	—	—	0.046	—	0.130	0.200	0.245	0.331
	"	0.2%	0.05%	—	0.046	—	0.132	0.174	0.198	0.221
I	"	—	—	—	0.046	0.109	0.168	0.256	0.345	0.491
	"	0.2%	—	—	0.046	0.110	0.166	0.256	0.316	0.351
	"	0.2%	—	0.05%	0.046	0.108	0.169	0.213	0.288	—
H	Dubos	—	—	—	0.046	—	0.132	0.172	0.228	0.243
	"	0.2%	—	—	0.046	—	0.139	0.191	0.222	0.239
	"	0.2%	—	0.05%	0.046	—	0.137	0.176	0.217	0.224
I	"	—	—	—	0.046	0.109	0.168	0.215	0.269	—
	"	0.2%	—	—	0.046	0.110	0.169	0.211	0.265	—
	"	0.2%	—	0.05%	0.046	0.103	0.166	0.196	0.234	—

as for the medium containing tween 80 alone, following which more growth was present in the medium containing both tween 80 and glycerol.

Graph 4 shows the results obtained using Dubos basal medium with and without glucose in the presence and absence of 0.05 per cent purified tween 80. The results obtained here are comparable to the results obtained with glycerol in the modified Proskauer and Beck synthetic medium.

The effect of albumin (0.2 per cent) in modified Proskauer and Beck and Dubos medium on the growth of tubercle bacilli (H37Rv). The addition of 0.2 per cent albumin to the basal media did not result in an increased rate of growth either in the early growth period or in the late phase. During the late growth period there was a tendency for an earlier decrease in rate of growth in the Proskauer and Beck medium containing albumin, but in all other respects the growth rate as well as the total amount of growth are essentially equal (graphs 5 and 6; table 2).

The effect of albumin (0.2 per cent) in the presence of tween 80 (0.05 per cent) on the growth of tubercle bacilli (H37Rv). The addition of tween 80 (0.05 per cent) in either the purified or unpurified state produced no significant differences in the rate of growth in Dubos medium or modified P and B medium in the presence of 0.2 per cent albumin for the first 5 days, but thereafter a decrease in growth was noted in both media which contained either purified or unpurified tween 80 (graphs 5 and 6; table 2).

DISCUSSION

It is apparent from the results of these experiments that unpurified tween 80 has a rather marked bacteriostatic effect on the large inocula of tubercle bacilli employed. This is in line with the observations of Dubos and Davis (1946) and Davis and Dubos (1946a) that this material is toxic to small inocula of tubercle bacilli. According to Dubos and Davis (1946a), Dubos, Davis, Middlebrook,
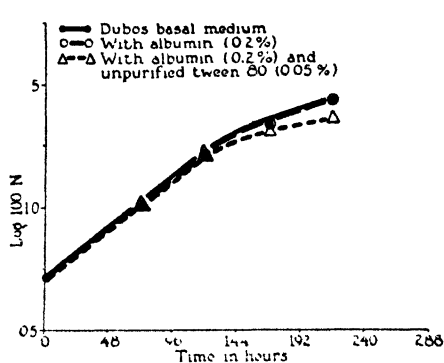
Graph 5, left. Effect of serum albumin (0.2 per cent) and unpurified tween 80 (0.05 per cent) on growth of *M. tuberculosis* (H37Rv) in Dubos basal synthetic medium.
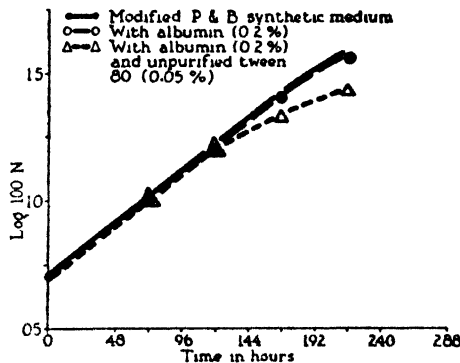
Graph 6, right. Effect of serum albumin (0.2 per cent) and unpurified tween 80 (0.05 per cent) on growth of *M. tuberculosis* (H37Rv) in modified P and B synthetic medium.

and Pierce (1946), and Dubos and Middlebrook (1947), the toxicity of this material is due to the presence of unesterified oleic acid; and, when the oleic acid is chemically removed from the tween 80, the latter becomes entirely nontoxic for tubercle bacilli as determined by the growth of small inocula. Our results would indicate that tween 80 from which the free oleic acid has been removed still has a bacteriostatic action even on large inocula of tubercle bacilli. This, of course, might indicate that purification of the tween 80 had not been complete. The protective effect of bovine albumin noted in our experiments might also suggest that our purified tween 80 was not completely free of unesterified oleic acid. According to Davis (1947), purified tween 80 will slowly hydrolyze at 37 C to produce some free oleic acid, and it is possible that our findings could be accounted for on this basis. In view of the slow rate of hydrolysis of tween 80 and the prompt inhibition of growth observed in our experiments this explanation does not seem likely.

According to Dubos and Middlebrook (1947), lipases present in bacteria may accelerate the hydrolysis of tween 80. In view of the relatively large inocula employed in these experiments the possibility must be considered that the tubercle bacilli themselves might have hydrolyzed the tween 80 to form oleic acid with a consequent bacteriostatic effect. According to Dubos (1947) and Davis (1948), however, the toxicity of oleic acid can be neutralized by large inocula of tubercle bacilli. This would appear to exclude the size of the inoculum as a factor.

It would seem, exclusive of the role played by any free oleic acid present in tween 80, that this substance by virtue of its surface-active properties might possibly be bacteriostatic per se. In any event, in the media employed and under the conditions of the experiments, there is no indication of the marked enhancement of growth reported by Dubos (1947). In this connection it is of interest to note that cultures of tubercle bacilli in media containing tween 80 usually appear visually, because of the diffuse type of growth, to have more organisms present; but in all cases, except when the glucose or glycerol were omitted from the media, in which actual quantitative measurements of the amount of tubercle bacilli present were made, a greater total mass of organisms was found in the media that were without tween 80 and that showed the clumped form of growth.

It is also apparent from these results that both glycerol and glucose markedly stimulate growth. Furthermore, purified tween 80 also stimulates growth when added to either of the media in the absence of glucose or glycerol, but not to nearly the same degree as either glucose or glycerol alone. Purified tween 80 when added to either of the media in the presence of glycerol or glucose inhibited growth as compared with either glucose or glycerol alone. This is in contrast to the findings of Dubos and Davis (1946) and Dubos, Davis, Middlebrook, and Pierce (1946) that glycerol inhibited the growth of small inocula in the presence of tween 80; actually the converse held in our experiments, since the tween 80 inhibited growth in the presence not only of glycerol but also of glucose, even though it stimulated growth slightly in the absence of these agents.

Although it is possible that glycerol might have an inhibitory effect on small numbers of tubercle bacilli and stimulate growth of larger numbers, it would also seem reasonable that another substance (tween 80) that was toxic for large inocula might also have a growth-retarding effect on small inocula. This is supported by work using small inocula (Youmans, 1948).

Under the conditions of these experiments, using large inocula, the addition of serum albumin (0.2 per cent) resulted in neither an increased initial rate of growth nor an increased total yield. According to Dubos and Davis (1946) and Davis and Dubos (1947), although the addition of crystalline serum albumin to the medium often permits the initiation of growth of minute inocula that would not grow in the absence of the protein, it does not increase appreciably the final density of the culture. However, less pure protein (fraction V) apparently contained heat-stable impurities that could be separated from the albumin and that increased markedly the amount of growth yielded by tubercle bacilli in

synthetic media. In our experiments no such enhancing effect resulted from the addition of fraction V serum albumin to the basal medium. In fact, during the late growth period there was a tendency toward a slight decrease in rate of growth in the Proskauer and Beck medium containing albumin, but the initial growth rates were not significantly different. These divergent results, of course, might be accounted for on the basis of differences in the samples of bovine albumin employed.

The addition of purified and unpurified tween 80 to either medium containing 0.2 per cent albumin resulted in no significant difference in the rate of growth for the first 5 days; thereafter, a decrease in the rate of growth was noted which was more marked in the modified Proskauer and Beck medium. Thus it appears that the albumin protected against the early inhibitory effect of tween 80 but not for a period greater than 5 days. The combined bovine serum albumin and tween 80 mixture definitely failed to increase either the rate of growth or total yield as compared to the results when using only Dubos basal medium or modified Proskauer and Beck synthetic media.

Though the role of these various agents as tested seems definite, it does not necessarily follow that under conditions in which different concentrations were used the same results would be noted. Because of the laboriousness of this quantitative method the present experiments were limited to the use of these substances in the concentrations recommended in the literature. The answer to questions relative to the effect of varying the concentrations must await further quantitative study. Furthermore, it cannot be concluded that all strains of virulent human type tubercle bacilli would behave under the conditions of the experiments in the same manner as the H37Rv strain.

CONCLUSIONS

Unpurified "tween 80" (0.05 per cent) markedly inhibited the growth rate of virulent tubercle bacilli (H37Rv) in modified Proskauer and Beck synthetic media.

Purified tween 80 (0.05 per cent) exerted a similar bacteriostatic effect but to a lesser degree.

The same inhibitory effect of this surface-active agent was noted using Dubos basal medium.

Glucose and glycerol markedly stimulated the rate of growth.

Although purified tween 80 inhibited growth in the presence of glucose and glycerol, it stimulated growth slightly in the absence of these agents.

Bovine serum albumin (fraction V; 0.2 per cent) did not stimulate growth of the tubercle bacillus.

Bovine serum albumin (0.2 per cent) protected tubercle bacilli against the inhibitory effect of purified and unpurified tween 80 (0.05 per cent) but only during the first 5 days of growth.

The modified P and B synthetic medium containing 2.0 per cent glycerol supported growth of tubercle bacilli at a maximum rate for a longer period than the Dubos medium containing 0.2 per cent glucose.

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THE EFFECT OF "TWEEN 80" IN VITRO ON THE BACTERIOSTATIC ACTIVITY OF TWENTY COMPOUNDS FOR MYCOBACTERIUM TUBERCULOSIS[1]

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The use of tween-albumin medium for the cultivation of tubercle bacilli as recommended by Dubos and Davis (1946) affords the advantage of permitting a relatively diffuse homogeneous growth instead of the granular, flocculent growth usually encountered. This permits greater ease of handling and the use of more uniform inocula. The incorporation of "tween 80" and bovine albumin in media to be used for the *in vitro* study of bacteriostatic and bactericidal agents on tubercle bacilli has been recommended by several workers (Duca, Williams, and Scudi, 1948; Middlebrook and Yegian, 1946; Dubos and Middlebrook, 1947; Wong, Hambly, and Anderson, 1947). However, it has been reported that the presence of tween 80 in synthetic media increased the bacteriostatic action of penicillin (Kirby and Dubos, 1947), streptomycin (Fisher, 1948), and subtilin (Anderson and Wong, 1946; Anderson, 1947) on the subsurface growth of tubercle bacilli. Bloch, Erlenmeyer, and Suter (1947) and Forrest, Hart, and Walker (1947) found, on the contrary, that tween 80 reduced the inhibitory action of certain compounds on the growth of this organism. As a result of these contradictory reports, this work was undertaken to determine what effect tween 80, in the presence and absence of bovine albumin, would have on the *in vitro* bacteriostatic activity of 20 compounds for *Mycobacterium tuberculosis*.

EXPERIMENTAL METHODS

The basal medium and methods used were essentially those recommended by Youmans (1944) and Youmans and Doub (1946). The basal medium employed had the following composition:

Asparagine	5.0 g
KH_2PO_4	5.0 g
K_2SO_4	0.5 g
Magnesium citrate	1.5 g
Glycerol	20.0 ml
Double distilled water	1,000.0 ml

The pH of the medium was adjusted to 7.0 with 40 per cent NaOH, and the medium was autoclaved then at 15 pounds for 20 minutes.

Twofold dilutions of each compound were used in the basal medium and each of the following modifications: (a) basal medium plus 0.2 per cent crystalline bovine albumin,[2] (b) basal medium plus 0.05 per cent tween 80, (c) basal medium

plus 0.05 per cent tween 80 plus 0.2 per cent bovine albumin, (d) basal medium plus 0.05 per cent purified tween 80, and (e) basal medium plus 0.05 per cent purified tween 80 plus 0.2 per cent bovine albumin.   The media containing the concentrations of each drug, which varied from 10 mg per cent to 0.00002 mg per cent, were inoculated with 0.1 mg of a very fine suspension of the H37Rv strain of *M. tuberculosis* var. *hominis*.   After incubation at 37 C for 2 weeks the tubes were examined, and the lowest concentration of the compound that completely inhibited the subsurface growth of the organism was considered the bacteriostatic level of that compound.   Diffuse growth always was obtained in the presence of tween 80.

The two samples of tween 80[3] (lot nos. Arl. 3292 and 8349) employed in these experiments were obtained from the Atlas Powder Company and were from stock recommended for the growth of tubercle bacilli.   The purified tween 80, from which the free oleic acid was removed, was prepared according to the method of Davis (1947).[4]   Fresh solutions of these were prepared for each batch of medium.

RESULTS

The results are tabulated in table 1, and are the averages of from 3 to 6 separate determinations on each compound except when purified tween 80 was employed. In the latter instance only 1 to 2 determinations were made because of the small supply of purified tween 80.

In comparison of the results of the bacteriostatic activities of the compounds on the tubercle bacilli when the basal medium alone was employed with those when tween 80 was added to the medium, it was noted that, in the presence of tween 80, 15 out of 20 compounds were more bacteriostatic.   Two of the 20 compounds (nos. 8 and 16) were almost equally bacteriostatic in both media, whereas 3 compounds (nos. 14, 15, 17) produced much less inhibition of growth when tween 80 was added to the medium.

Albumin added to the basal medium, with the possible exception of 7 compounds (nos. 1, 2, 3, 4, 18, 19, 20), decreased the bacteriostatic activity of the compounds.   An analysis of the effect of tween 80 in the presence of albumin on the bacteriostatic end points of the compounds was difficult, in that albumin alone decreased the tuberculostatic effect of most of the compounds, but tween 80 alone increased it.   In most cases, however, it might be assumed that the albumin counteracted the effect of tween 80 since, in the tween-albumin medium, only 5 compounds (nos. 1, 2, 3, 18, 20) were more bacteriostatic than they were in the basal medium alone.   Four compounds (nos. 4, 7, 12, 19) gave approximately the same results, but the remaining 11 compounds were less bacteriostatic than in the basal medium.

In the presence of purified tween 80, 7 of the 14 compounds tested were less bacteriostatic than in the presence of unpurified tween 80.   The remaining 7 compounds inhibited bacterial growth approximately the same in both media.

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[3] Polyoxyethylene sorbitan monooleate.

[4] Prepared by Leonard Doub of the Parke, Davis Research Laboratories, Detroit, Michigan.

The bacteriostatic activity of 12 of the 13 compounds tested was essentially the same in both the purified tween-albumin medium and in the unpurified tween-albumin medium.

To determine whether the effects produced by tween 80 on the bacteriostatic activity of compounds for *M. tuberculosis* could be reduced by decreasing the

TABLE 1

The effect of tween 80 in the presence and absence of 0.2 per cent crystalline bovine albumin, on the tuberculostatic activity of various compounds

NO.	COMPOUND	B.M.*	B.M. + ALBUMIN	B.M. + TWEEN 80	B.M. + TWEEN 80 + ALBUMIN	B.M. + PURIFIED TWEEN 80	B.M. + PURIFIED TWEEN 80 + ALBUMIN
1	Sulfanilamide....	1.0†	1.0	6.6	4.0		
2	Sulfapyridine..	1.0	1.0	16.0	8.0		
3	Sulfathiazole	1.0	0.82	8.6	8.6	8.6	8.6
4	4-Allylamino-4'-amino-diphenyl sulfone..	1.0	0.83	6.2	0.8	6.2	0.8
5	2-Nitro-5-sulfanilyl thiophene. .	1.0	0.47	9.6	0.73		
6	2,2'-Dinitro-5,5'-dithienyl sulfone.	1 0	0.49	2.7	0.34	2.7	0.34
7	4,4'-Diamino diphenyl sulfone....	1.0	0.75	10.4	1.4	2.7	0.88
8	2-Methyl naphthoquinone	1.0	0.47	1.3	0.16	0.36	0.03
9	p-Xyloquinone....	1.0	0.5	2.2	0.66		
10	1,4-Naphthoquinone....	1.0	0.21	2.9	0.27		
11	2-Chloro-1,4-naphthoquinone.....	1.0	0.54	2.4	0.56	1.4	
12	8-Hydroxyquinoline... . . .	1.0	0.47	4.4	1.0	1.2	0.78
13	2,6-Dibromo-3,5-dimethyl-4-chloro phenoxyacetic acid......	1.0	0.7	3.2	0.70	1.2	0.90
14	p-Aminoazobenzene..	1.0	0.24	0.3	<0.20	0.26	<0.20
15	2(4'-Aminophenyl)pyridine.......	1.0	<0.0035	0.0053	<0.0035	<0.0035	<0.0035
16	N-p-aminophenyl-piperidine. .. .	1.0	0.5	1.3	0.41	0.75	0.37
17	4'-Chloro-4-aminodiphenyl sulfide	1.0	0.1	0.1	0.07	0.075	0.056
18	4-Amino-2-hydroxybenzoic acid hydrochloride.	1.0	0.95	18.5	15.0	18.5	15.0
19	β-(Indole-3)-propionic acid.... .	1.0	0.8	3.6	1.1	1.0	1.0
20	Chloromycetin...................	1.0	1.0	8.0	4.0		

* Basal medium.

† The figures in this table and subsequent tables indicate the bacteriostatic index which was found by dividing the lowest bacteriostatic concentration of each compound in the basal medium by the lowest bacteriostatic concentration obtained in each of the other media; therefore, the values above 1.0 indicate greater bacteriostasis, whereas the values below 1.0 indicate less bacteriostasis than found in the basal medium.

concentration, smaller amounts of tween 80 were employed both with and without 0.2 per cent albumin in the medium. Five compounds were chosen which, in the presence of tween 80, consistently produced different tuberculostatic end points than were found in the basal medium alone. The results are found in table 2. A 4-fold decrease in the amount of tween 80 in the basal medium did not markedly affect the bacteriostatic end points.

Since tween 80, according to Davis and Dubos (1946), may contain as much as 0.6 per cent by weight of free oleic acid, or 0.3 mg per cent oleic acid in the medium when tween 80 is employed in a concentration of 0.05 per cent, 4 concentrations of oleic acid, in the presence and absence of albumin, were added to

TABLE 2

The effect of smaller concentrations of tween 80, in the presence and absence of 0.2 per cent crystalline bovine albumin, on the tuberculostatic activity of 5 compounds

COMPOUND	B.M.*	B.M. + ALBUMIN	CONCENTRATION OF TWEEN 80 IN PER CENT						
			B.M. + tween 80 (0.05)	B.M. + tween 80 (0.05) + albumin	B.M. + tween 80 (0.025)	B.M. + tween 80 (0.025) + albumin	B.M. + tween 80 (0.0125)	B.M. + tween 80 (0.0125) + albumin
Sulfathiazole...................	1.0	1.0	8.0	8.0	8.0	8.0	8.0	8.0
2,2'-Dinitro-5,5'-dithienyl sulfone............	1.0	0.25	2.0	0.5	1.0	0.25	1.0	0.25
4-Allylamino-4'-amino-diphenyl sulfone..	1.0	1.0	4.0	2.0	4.0	2.0	4.0	2.0
2(4'-Aminophenyl)pyridine...	1.0	0.00184	0.0036	0.00184	0.0009	0.00184	0.0009	0.00184
4-Amino-2-hydroxybenzoic acid hydrochloride........ .	1.0	1.0	17.0	17.0	8.0	8.3	8.3	8.3

' Basal medium.

TABLE 3

The effect of varying concentrations of oleic acid, in the presence and absence of 0.2 per cent crystalline bovine albumin, on the tuberculostatic activities of 5 compounds

COMPOUND	B.M.*	B.M. + ALBUMIN	CONCENTRATION OF OLEIC ACID IN MILLIGRAMS PER CENT						
			B.M. + oleic acid (0.6)	B.M. + oleic acid (0.6) + albumin	B.M. + oleic acid (0.3)	B.M. + oleic acid (0.3) + albumin	B.M. + oleic acid (0.15)	B.M. + oleic acid (0.15) + albumin	B.M. + oleic acid (0.075) + albumin
Sulfathiazole	1.0	1.0	No growth	1.0	1.0	1.0	2.0	1.0	2.0	1.0
2,2'-Dinitro-5,5'-dithienyl sulfone	1.0	0.625	No growth	0.125	32.9	0.125	16.0	0.5	8.01	0.5
4-Allylamino-4'-amino-diphenyl sulfone	1.0	0.5	No growth	1.0	8.0	0.5	1.0	0.5	1.0	0.5
2(4'-Aminophenyl)pyridine	1.0	0.00092	No growth	0.00046	0.48	0.00046	1.0	0.00092	1.0	0.00092
4-Amino-2-hydroxybenzoic acid hydrochloride	1.0	1.0	No growth	2.0	35.4	2.0	4.1	2.0	2.0	2.0
None.................	Good growth	Good growth	No growth	Good growth	Slight growth	Good growth	Good growth	Good growth	Good growth	Good growth

' Basal mediun

the basal medium and tested with each of the foregoing 5 compounds against H37Rv. This was done to determine whether free oleic acid might be the substance in tween 80 responsible for the observed alteration in bacteriostatic activity. The results are shown in table 3.

As indicated in table 3, 0.3 mg per cent oleic acid or less had no effect on the

tuberculostatic action of sulfathiazole and 2(4'-aminophenyl)pyridine; this was in contrast to the results found with tween 80 incorporated in the medium. Oleic acid in 0.3 mg per cent concentration increased the bacteriostatic action of 4-allylamino-4'-aminodiphenyl sulfone and 4-amino-2-hydroxy-benzoic acid hydrochloride to a degree similar to that noted upon the addition of tween 80 to the basal medium.   The 0.3 mg per cent concentration of oleic acid increased the bacteriostatic action of 2,2'-dinitro-5,5'-dithienyl sulfone 32-fold as compared to a 2-fold increase produced by tween 80.   As the concentration of the oleic acid was decreased by half, the bacteriostatic action of this compound was reduced one-half.

DISCUSSION

It is apparent from these results that both unpurified and purified tween 80 increased the bacteriostatic activity of many of the compounds, did not affect the action of some, and reduced the bacteriostatic activity of a few.   The addition of albumin alone to the basal medium reduced the bacteriostatic action of the majority of the compounds, but did not increase the degree of bacteriostasis of any.   In the presence of albumin, unpurified tween 80 still markedly increased the bacteriostatic activity of 5 compounds (nos. 1, 2, 3, 18, 20), none of which was affected by the presence of albumin alone, and notably decreased the inhibition of growth of 3 compounds (nos. 14, 15, 17).   Unpurified tween 80 and purified tween 80 gave approximately the same results with 7 out of the 14 compounds tested.   With the remaining 7 compounds, however, purified tween 80 produced less inhibition of bacteriostasis than the unpurified tween 80.

The bacteriostatic activity of chloromycetin was increased in the presence of tween 80, and this activity was only slightly decreased by albumin.   This agrees with the results found with three other antibiotics: streptomycin (Fisher, 1948), penicillin (Kirby and Dubos, 1947), and subtilin (Anderson and Wong, 1946; Anderson, 1947; Youmans, 1948).

Bloch, Erlenmeyer, and Suter (1947) in testing 7 compounds found that tween 80 decreased their bacteriostatic action on *M. tuberculosis*.   Sulfathiazole was the only compound of the 7 used by Bloch and his associates that was employed in this series of tests.   Contrary to Bloch's findings, however, tween 80 potentiated the inhibition of growth produced by sulfathiazole, and this action was not decreased by the addition of albumin to the medium.   Bloch and his associates also found that tween 80 decreased the inhibition of growth by streptomycin, which is in contrast to the findings of Fisher (1948).

Forrest, Hart, and Walker (1947) reported on one compound, 5-amino-2-butoxypyridine, the bacteriostatic activity of which was reduced by the presence of tween 80.   The same effect was noted with one pyridine compound, 2(4'-aminophenyl)pyridine, which was employed in this experiment.

The explanation for the divergent results obtained with tween 80 on the 20 compounds employed in this study is not apparent.   There appears to be no relationship between the action of tween 80 on these compounds and their chemical structures, nor did a 4-fold reduction in the concentration of tween 80 produce significant changes in the results with 5 compounds.

According to Davis and Dubos (1946), tween 80 may contain as much as 0.6 per cent by weight of free oleic acid. Oleic acid alone, however, had no effect on the tuberculostatic activity of sulfathiazole or on 2(4'-aminophenyl)pyridine, although tween 80 had increased 8-fold the inhibition of growth caused by sulfathiazole, and had decreased the bacteriostatic action of 2(4'-aminophenyl)pyridine more than 1,000-fold. Thus, oleic acid could hardly be the substance in tween 80 responsible for these results. Oleic acid tested with 4-allylamine-4'-aminodiphenyl sulfone and with 4-amino-2-hydroxy-benzoic acid hydrochloride increased their bacteriostatic activity to a degree similar to that obtained on the addition of tween 80, so that, if present in unpurified tween 80, it may have been responsible for the greater inhibition of growth observed with tween 80. However, purified tween 80 had the same effect as unpurified tween 80 on these compounds. Moreover, decreasing the concentration of tween 80 should lower the concentration of free oleic acid, if present, with a consequent reduction of the tween effects. This did not occur. These findings indicated that free oleic acid probably did not account for the observed results. The 0.3 mg per cent concentration of oleic acid increased 32-fold the inhibition of growth produced by 2,2'-dinitro-5,5'-dithienyl sulfone, although tween 80 had increased it only 2-fold. Since this compound appeared to be quite sensitive to the action of oleic acid, and only slightly sensitive to the action of tween 80, the possibility should be considered whether the tween 80 "masked" the effect of the free oleic acid that might be present in tween 80. Since similar results were obtained with both purified tween 80 and unpurified tween 80, and in view of the results obtained with the other 4 compounds, this would appear unlikely. Furthermore, since albumin neutralizes the action of oleic acid (table 3), the increased bacteriostasis produced by tween 80 (table 1) in the presence of albumin could not be due to free oleic acid.

The mechanism whereby tween 80 produces either increased or decreased bacteriostasis, depending upon the compound, is not clear. It is possible that the former effect may be a function of its surface-active properties, permitting a more intimate contact between the drug and the surface of the bacterial cell (Kirby and Dubos, 1947). In this connection, however, it should be noted that tween 80 is also bacteriostatic for tubercle bacilli (Sattler and Youmans, 1948), and the increased bacteriostasis with some of the compounds in the presence of tween 80 might be due to a combination of bacteriostatic effects. Since the bacteriostatic activity of some compounds is also markedly reduced by tween 80, the tween 80 may combine with these compounds to reduce the effective concentration. In this respect it would function in a manner similar to the albumin.

The irregular results produced by the presence of tween 80 on the tuberculostatic activity of compounds indicate the desirability of employing as simple a chemically defined synthetic medium as is possible for the growth of the bacteria. This is necessary in order to analyze accurately the relative bacteriostatic activity of compounds. In conjunction with this test, plasma, serum, or albumin can be added to the synthetic medium of a second test to determine the inactivation of the compound by protein.

CONCLUSIONS

"Tween 80" in a synthetic medium, as compared to the synthetic medium alone, increased the tuberculostatic activity of 15 out of 20 compounds, decreased the activity of 3 compounds, and had no effect on 2 compounds.

Crystalline bovine albumin decreased the inhibition of growth of 13 out of the 20 compounds.

In the tween-albumin medium, 5 compounds were more bacteriostatic than in the basal medium, 4 gave approximately the same results in the two media, and the remaining 11 were less bacteriostatic than in the basal medium.

In the presence of purified tween, 7 of the 14 compounds were less bacteriostatic than in the presence of unpurified tween 80. The remaining 7 compounds inhibited bacterial growth approximately the same amount in both media.

The bacteriostatic activity of 12 of the 13 compounds was essentially the same in both the purified tween-albumin medium and in the unpurified tween-albumin medium.

Decreasing the concentration of tween 80 4-fold in the medium did not alter significantly the bacteriostatic levels of the 5 compounds tested.

Under the conditions of the experiments the free oleic acid that may be present in tween 80 apparently was not responsible for the effect of tween 80 on the bacteriostatic activities.

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THE REVERSAL OF BENZIMIDAZOLE INHIBITION OF GROWTH BY NUCLEIC ACID[1]

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The inhibitory effect of benzimidazole on the growth of *Escherichia coli* has been reported previously by Woolley (1944), who found also that the inhibition could be reversed by adenine. This behavior led Woolley to suggest that the action of benzimidazole involves an interference with the purine metabolism of the microorganism.

The close relation between the purines and nucleic acids prompted us to examine the effect of yeast nucleic acid on the inhibitory activity of benzimidazole against *E. coli.* Marked reversals are obtained as is evident from the data in table 1. For example, a comparison of the relative growth in group III A with that obtained in group I in the presence of 10 mg per cent benzimidazole and no nucleic acid shows an increase in growth ranging from 55 to 154 per cent.

This reversal by yeast nucleic acid cannot be due merely to its content of adenine, for the effect is observed at much lower concentrations. Woolley reported adenine reversals at about 60 mg per cent concentrations. Our experiments at lower concentrations, 5 to 20 mg per cent, comparable with those at which marked reversals of inhibition are obtained with nucleate, showed no reversal by the purine. In fact, the presence of the purine seemed to increase the inhibitory effect of the benzimidazole (table 1, group VA).

It has been observed also that the nucleic acid has a stimulating effect on the growth of *E. coli* in the synthetic medium of MacLeod (1940). Stimulation by nucleic acids and their hydrolyzates has been observed in several other situations, for example, by Brewer *et al.* (1946) in *Bacillus anthracis.*

To obtain a further insight into the nature of the antagonism between benzimidazole and nucleate, quantitative studies of growth rates have been made at various concentrations of the stimulant and inhibitor. These data have been analysed by the Lineweaver and Burk (1934) method to determine the nature of the inhibition. Logarithms of the turbidity readings have been plotted against time; and the slopes of the linear region, the time interval during which logarithmic growth was obtained, have been used as a relative measure of the rate constant. The reciprocals of these rate constants for various concentrations of benzimidazole have been plotted in figure 1 against the reciprocals of the concentration of the nucleate. The progressive increase in slope of the lines in figure 1 with increase in concentration of benzimidazole, together with the strong indication of a common intercept, leads one to conclude that the inhibition of nucleate by benzimidazole is a competitive one.

TABLE 1
Effects of various substances on the growth of E. coli

| | CONCENTRATION, MG PER CENT | | | RELATIVE GROWTH AT 9 HR |
	Benzimidazole	Nucleic acid	Adenine	
Control	—	—	—	1.00
I	60	—	—	0.03
	25	—	—	0.17
	10	—	—	0.56
	5	—	—	0.82
II	—	20	—	1.61
	—	10	—	1.48
	—	5	—	1.04
III A	10	20	—	1.42
	10	10	—	1.34
	10	5	—	0.87
B	60	20	—	0.13
	60	10	—	0.09
	60	5	—	0.05
IV	—	—	20	0.72
	—	—	10	0.79
	—	—	5	0.78
V A	10	—	20	0.25
	10	—	10	0.38
	10	—	5	0.50
B	60	—	20	0.03
	60	—	10	0.03
	60	—	5	0.03
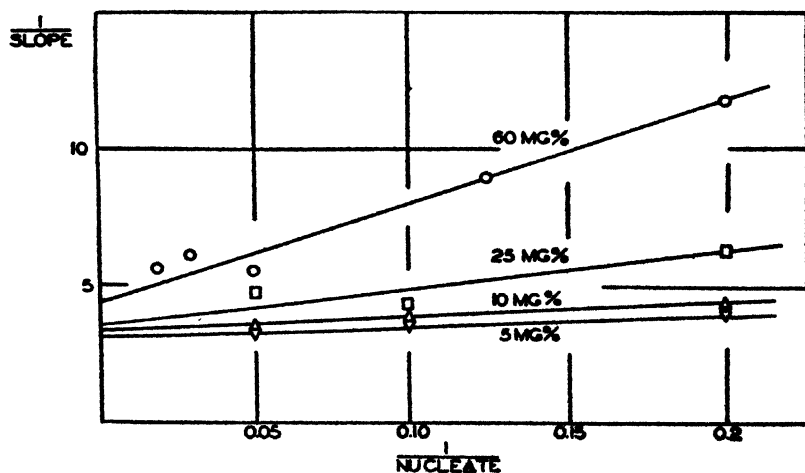
Figure 1. Competitive inhibition of nucleic acid by benzimidazole in growth of E. coli in synthetic medium (2). Each line corresponds to a given concentration of benzimidazole indicated in mg per cent.

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NOTE

GROWTH INHIBITION OF THE H37 STRAIN OF HUMAN TUBERCLE BACILLI BY β-2-THIENYLALANINE AND ITS PREVENTION BY PHENYLALANINE

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β-2-Thienylalanine has been shown to inhibit the growth of the rat and microorganisms by du Vigneaud and coworkers (J. Biol. Chem., **159**, 385). They demonstrated that the growth inhibition could be prevented by phenylalanine. These results have been confirmed by Dittmer *et al.* (J. Biol. Chem., **164**, 761)

TABLE 1

Effect of thienylalanine on tubercle bacilli

EXPERI-MENT NO.	TOTAL VOLUME PER FLASK	β-2-THIENYLALANINE ADDED PER FLASK	RESULTS IN GROWTH
	ml	mg	
	20	20	No growth in 60 days
	20	2	No growth in 12 days; slight growth at 34 days
1	20	0.2	Normal growth, no inhibition
	20 Normal controls	0	Some growth in 8 days; maximum growth in 34 days
	20	20	No growth in 60 days
	20	20 plus 19.2 mg of DL-phenylalanine	Some growth in 7 days; maximum growth in 39 days
3	20	10	Very slight growth in 60 days
	20	10 plus 9.6 mg of DL-phenylalanine	Some growth in 7 days; maximum growth in 39 days
	20 Normal controls	0	Some growth in 7 days; maximum growth in 39 days

and Beerstecher and Shive (J. Biol. Chem., **164**, 53). Thompson (J. Immunol., **55**, 345) reported that amino acid antagonists prevent the normal development of vaccinia virus. These results suggested an investigation of the effects of amino acid antagonists on the growth and virulence of the human tubercle bacillus. In this paper are reported the effects of β-2-thienylalanine on the *in vitro* growth of the H37 strain of human tubercle bacilli.

The technique used in these tests was that previously described by Drea

(Am. Rev. Tuberc., **53,** 353), which consisted of adding increasing amounts of β-2-thienylalanine to Long's synthetic medium which, after inoculation, permitted, in the controls, earliest bottom growth in 8 days and profuse surface growth in 34 days.

A concentration of 20 mg of β-2-thienylalanine in 20 ml of medium prevented all growth for 60 days. Lower concentrations of the antagonist had less or no effect on the normal growth of the tubercle bacilli.

When phenylalanine was added together with bacteriostatic concentrations of thienylalanine, normal growth was obtained. Typical experimental results are given in table 1.

SUMMARY

β-2-Thienylalanine inhibited the growth of the H37 strain of human tubercle bacilli, and this inhibition was prevented by added amounts of phenylalanine. These results indicate that the growth of the tubercle bacilli can be affected by interfering with the metabolism of amino acids.

STREPTOMYCES GRISEUS (KRAINSKY) WAKSMAN AND HENRICI[1]

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Since the announcement of the isolation of streptomycin, an antibiotic substance produced by certain strains of *Streptomyces griseus* (Schatz, Bugie, and Waksman, 1944), an extensive literature has accumulated dealing with this antibiotic and with the organism producing it. Although most of the investigations are concerned with the production, isolation, and chemical purification of streptomycin, its antimicrobial properties, and especially its utilization as a chemotherapeutic agent, various reports are also devoted to the isolation of new streptomycin-producing strains of *S. griseus* and of other actinomycetes and to the development of more potent strains. Only a very few of the cultures of *S. griseus* that have been isolated from natural substrates, following the islation of the two original cultures in 1943, were found capable of producing streptomycin. One such culture was reported from this laboratory (Waksman, Reilly, and Johnstone, 1946), and one or two from other laboratories (Carvajal, 1946*a,b*). It has been shown more recently (Waksman, Reilly, and Harris, 1947) that by the use of selective methods other streptomycin-producing strains can easily be isolated and identified.

In addition to *S. griseus*, certain other actinomycetes are able to form streptomycin or streptomycinlike substances. One such culture was described by Johnstone and Waksman (1947) as *Streptomyces bikiniensis,* another culture was isolated by Trussell, Fulton, and Grant (1947) and was found capable of producing a mixture of two antibiotics, one of which appeared to be streptomycin and the other streptothricin.

HISTORICAL REVIEW

The first isolation of an organism described as *Actinomyces griseus* was reported by Krainsky (1914), who obtained this culture from a Russian soil. This report appeared at the outbreak of the First World War, and the culture itself was, therefore, not available for comparative studies in this country. When soon afterward a similar culture was isolated from an American soil by Waksman and Curtis (1916), the comparative studies had to be based entirely upon the description of the culture made by Krainsky. Although the cultural characteristics of the new isolate appeared to correspond closely to those given for the original culture, doubt was expressed as to the identity of the two cultures, as indicated by the statement that "the color of the aerial mycelium is somewhat lighter than that described by Krainsky."

The culture isolated by Waksman and Curtis grew readily upon synthetic media, producing an olive-buff vegetative growth. The aerial mycelium ap-

peared at an early stage in the form of a thick powdery mass of a water-green color. Microscopically, this mycelium was found to consist of long filaments with very little branching; these fragmented readily into rod-shaped conidia, 1 to 1.5 by 0.8 μ. Heavy growth was produced in liquid glucose media. It consisted of round colonies (1 to 3 mm in diameter) floating on the surface of the medium, with a powdery white aerial mycelium covering the growth.

In a further study of the distribution of actinomycetes in various soils, Waksman and Curtis (1918) isolated *S. griseus* from three other American soils obtained from California, Oregon, and Texas. Because the ability of actinomycetes to produce antibiotic substances was not recognized at that time, these cultures were not tested either for their capacity to inhibit the growth of bacteria or of other organisms or to produce substances that had such capacity.

As will be shown later, the streptomycin-producing strains of *S. griseus* give rise to two kinds of variants or mutants, one of which does not produce any antibiotic and the other produces an antibiotic that is distinct from streptomycin. This suggests the possibility that the original New Jersey culture may have had the capacity of producing streptomycin or another antibiotic but that this property was lost upon continued cultivation on artificial media, or that inactive variants or variants possessing a different kind of activity were produced during these three decades of artificial cultivation of this culture.

One of the most important characteristics of *S. griseus* is its marked variability when it is grown on artificial media. This was recognized in the early studies of this organism, as is brought out by the following quotation (Waksman, 1919):

Even more striking than the morphological are the physiological variations. These depend chiefly on the substratum of the mother culture, temperature and length of incubation, amount and kind of inoculum (vegetative or aerial mycelium or spores). For example, a certain culture (*A. griseus*) may at one time clot the milk at 37° in 2 days and then peptonize it (dissolve the clot) in 5–6 days; at another time, the same culture, under the same conditions, will clot the milk only in 5–6 days, then peptonize it in 12–15 days; while at a third time, some tubes may not show any clot at all, and the milk is hydrolyzed (cleared up without any previous clot).

The variability of *S. griseus* is both quantitative and qualitative in nature. The quantitative differences can be expressed in the ability of different strains isolated from different substrates or of different substrains isolated from the same culture to produce varying amounts of streptomycin; quantitative variations are also observed in the characteristics of the organisms, such as degree of liquefaction of gelatin, shade of pigmentation of aerial mycelium, etc. The qualitative differences are varied in nature, as is shown by the preferential ability of certain strains to grow under submerged conditions of culture and of others to grow under surface conditions, by the nature of the aerial mycelium, by the pigmentation of the vegetative growth, and by the ability of different strains to produce antibiotic substances.

From the point of view of their ability to produce antibiotic substances, certain variations among the different strains of *S. griseus* may be summarized briefly as follows:

(1) Some, if not most, of the strains of *S. griseus* isolated from natural substrates do not produce any antibiotic at all (Waksman, Schatz, and Reynolds, 1946).

(2) Some of the antibiotic-producing strains form streptomycin as the major antibiotic; in addition, they also form other antibiotics, as the antifungal substance actidione and the anti-gram-positive bacterial agent present in the mycelium of the organism.

(3) Some of the antibiotic-producing strains of *S. griseus* form grisein as the major antibiotic.

(4) Some streptomycin-producing strains of *S. griseus* are more active under stationary and others under submerged conditions of growth; they vary greatly in their quantitative production of streptomycin.

(5) Streptomycin-producing strains of *S. griseus* give rise to at least two types of mutants. One mutant has lost the capacity to produce aerial mycelium and streptomycin (Schatz and Waksman, 1945). The other mutant produces a pink to red pigmented vegetative mycelium and yields an antibiotic that is distinct from streptomycin (Waksman, Reilly, and Johnstone, 1946).

(6) Different strains of *S. griseus* vary greatly in their sensitivity to actinophage. The phage active against streptomycin-producing strains of *S. griseus* is not active against any other strains of this organism (Reilly, Harris, and Waksman, 1947).

These variations in strain specificity may help to explain certain differences observed in the production of antibiotics by freshly isolated cultures of *S. griseus* or by closely related species of *Streptomyces*. The following experiments tend to throw further light upon these variations.

EXPERIMENTAL RESULTS

Effect of the addition of streptomycin to the medium upon the selection of active strains. The fact that streptomycin-producing cultures of *S. griseus* give rise to inactive variants and the fact that these variants are sensitive to streptomycin, the active cultures being resistant to this antibiotic (Schatz and Waksman, 1945; Waksman, Reilly, and Johnstone, 1946), suggested the possibility of utilizing the method of enriching plating media with streptomycin in order to eliminate inactive strains and to permit the isolation of more active streptomycin-producing strains.

Four cultures of *S. griseus* capable of forming streptomycin and originally isolated from soil or from other natural substrates were used for this purpose. These cultures were grown on agar slants, and various dilutions of the spore suspension were plated out, two media being used, one an ordinary nutrient agar and another agar enriched with 50 μg per ml of streptomycin. The plates were incubated at 28 C and the total numbers of colonies developing were counted. The results obtained (table 1) show that the cultures of *S. griseus* isolated from natural substrates vary greatly in the number of streptomycin-resistant colonies produced from spores. Two of the cultures contained a much greater percentage of resistant colonies than the other two cultures.

A number of colonies were picked from the plates containing the two agar

media, grown on agar surfaces, and tested for their ability to produce strepto-
mycin. The colonies developing on the streptomycin-enriched agar gave no
better streptomycin-producing cultures than the colonies picked from the strep-
tomycin-free nutrient agar plates. Some of the colonies, independent of the
medium from which they were isolated, gave more active strains than the parent
cultures; others gave less active strains. The differences obtained among the
various colonies were quantitative rather than qualitative. One culture (no.
3498) obtained from such a colony produced much less streptomycin under
stationary conditions than under submerged conditions of growth, as is brought
out in table 2.

Isolation of non-streptomycin-producing variants. The streptomycin-producing
cultures of *S. griseus* give rise to two types of variants, one of which is inactive
antibiotically and produces no aerial mycelium (Schatz and Waksman, 1945)

TABLE 1

Sensitivity of different streptomycin-producing S. griseus cultures to streptomycin

Colonies developing from similar spore suspensions on different media

CULTURE NO.	STREPTOMYCIN-FREE NUTRIENT AGAR	STREPTOMYCIN-CONTAINING NUTRIENT AGAR	PER CENT OF ORGANISMS RESISTANT TO 50 μg/ML OF STREPTOMYCIN
3463	3,000,000	97,000	3.2
3464	4,600,000	3,000,000	69.1
3480	3,150,000	90,000	2.9
3481	96,500,000	71,000,000	73.6

TABLE 2

Production of streptomycin by no. 3498 grown under submerged and stationary conditions

EXPERIMENT NO.	SUBMERGED GROWTH		STATIONARY GROWTH	
	2 days	6 days	6 days	8-13 days
1	43	65	21	12
2	22	80	<10	<10

and the other produces pink to red vegetative growth and an antibiotic substance
that is distinct from streptomycin. The second variant was obtained by plating
active cultures of *S. griseus* on agar plates and picking those colonies that form a
pink to cherry-red pigmented growth, notably on glucose asparagine agar. The
greenish-gray aerial mycelium of these strains does not differ much from that of
the typical *S. griseus* cultures. When the pigmented strains are grown on media
favorable for streptomycin production, no streptomycin is obtained. Another
antibiotic is produced, however, that differs greatly in its bacteriostatic spectrum
from that of streptomycin, especially in its lack of activity against *Escherichia
coli* and in its very high potency against *Staphylococcus aureus* (up to 10,000
dilution units per 1 ml of metabolite solution). Actinophage active against
streptomycin-producing strains of *S. griseus* has no effect upon the pink substrain.
The pink substrain was grown on media commonly used for the production of

streptomycin. Excellent growth, of a deep-red color, was obtained in submerged cultures. The filtrate had a high potency against gram-positive bacteria but not against *E. coli* or other gram-negative bacteria. The active substance was dissolved in ether and was found to be largely active against gram-positive bacteria.

Antibiotic activity of the 1915 S. griseus culture. The original strain of *S. griseus* isolated from a New Jersey soil in 1915 was available in two cultures: one that was kept for over 30 years in the New Jersey culture collection, where it has been grown continuously in synthetic media; and the other, a transfer of this culture, that was kept in the Centraalbureau voor Schimmelcultures in Baarn, Holland, where it was deposited in 1921 and where it was grown in organic media, such as potato agar. The New Jersey culture was tested recently several times for its ability to inhibit bacterial growth, but it showed no antibiotic

TABLE 3

Production of an antibiotic substance by different colony isolates from the 1915 culture of S. griseus kept at Baarn, 3326a

STRAIN NO.	DILUTION UNITS OF ACTIVITY AGAINST DIFFERENT BACTERIA							
	Stationary growth 12 days				Submerged growth 5 days			
	E. coli	B. subtilis	B. mycoides	S. aureus	E. coli	B. subtilis	B. mycoides	S. aureus
1	5	60	30	50	50	>1,000	100	60
2	5	150	225	275	<10	<10	<10	<10
3	5	150	300	250	0	0	0	0
4	20	200	800	750	0	0	0	0
5	20	125	400	600	0	0	0	0
6	20	150	500	300	<10	<10	12	15
7	15	125	350	250	<10	8	15	15
8	15	100	400	350	<10	12	20	28
9	15	1,000	250	600	40	500	225	600
10	15	150	350	300	<10	<10	8	15
11	40	200	750	750	<10	<10	<10	<10

activity. The Baarn culture, however, possessed definite antibacterial properties and produced an antibiotic substance in the medium. Our attention was directed to this culture by several European investigators who obtained it from the Baarn collection and, believing that they were working with one of the streptomycin-producing strains, grew the culture on a large scale. Although they found that the culture produced a considerable amount of some antibiotic substance, they were unable to isolate any streptomycin by the methods developed for the isolation of this antibiotic.

When recently received from Baarn, the culture was plated out, and a few individual colonies were picked and transferred to agar slants. The colony isolates were grown in a glucose peptone meat extract medium commonly used for the study of streptomycin production. These isolates differed greatly in their ability to produce an antibiotic substance, as is brought out in table 3. Although all the 11 isolates thus obtained were found to produce an antibiotic

when grown under stationary conditions, only 2 cultures formed such a substance under submerged conditions of growth. The activity of this antibiotic against *E. coli* was rather limited, although it was very active against various gram-positive bacteria.

The antibiotic substance produced by the active isolates from the Baarn culture possessed solubility properties distinctly different from those of streptomycin. Although the antibiotic was readily adsorbed on norite, it could not be removed from it by acidified alcohol, as is usually the case with streptomycin; it was soluble in organic solvents, although only a small part was removed, especially by ethyl acetate and by chloroform. This is brought out in table 4.

TABLE 4

Comparative bacteriostatic spectra of culture filtrates and isolated preparations from the original 1915 strain and the streptomycin-producing strains of S. griseus

PREPARATION	*E. coli*	*B. subtilis*	*B. mycoides*	*S. aureus*
S. griseus: Original 1915 culture, Baarn strain				
Culture filtrate, 1 ml...............	10	200	400	500
Total units in solution				
1,150 ml..	11,500	230,000	460,000	575,000
Ether soluble*	100	1,280	480	960
Ethyl acetate soluble	500	2,500	1,500	1,375
Chloroform soluble	180	2,400	600	1,800
Acid alcohol soluble	200	525	210	525
Total units recovered in all fractions	500	6,705	2,790	4,660
S. griseus: Streptomycin-producing culture 3463				
Culture filtrate, 1 ml...............	75	500	—	30
Total units in solution				
1,000 ml.. ...	75,000	500,000	—	30,000
Total units recovered				
Acid alcohol preparation†..	40,000	200,000	—	24,000

* Adsorbed on norite, treated with various solvents. Activity reported for total used in this experiment 1,150 ml.

† Adsorbed on norite, removed with acid-alcohol, treated with ether; more than 50 per cent of activity was recovered.

Production of different antibiotics by S. griseus. Different strains of *S. griseus* are thus shown to vary greatly in their capacity to produce antibiotic substances. Some of the strains produce streptomycin, others grisein, still others yield mixtures of these with other antibiotics. This was first pointed out by Carvajal (1946b), who emphasized the danger of designating an antibiotic substance as "streptomycin" merely because it is produced by a strain of *S. griseus* or because a strain of the latter causes inhibition of bacterial growth comparable to that of the streptomycin-producing strains of *S. griseus*.

On the basis of their ability to form antibiotic substances, the various strains of *S. griseus* that have so far been tested can be divided into four groups: (1)

streptomycin-producing strains; (2) grisein-producing strains; (3) strains producing antibiotics other than streptomycin or grisein, as in the case of the red-pigmented variant of the streptomycin strain described above or the Baarn variant of the 1915 culture; and (4) non-antibiotic-producing strains.

An attempt was made to establish whether any morphological or cultural differences existed among these four groups of cultures. The cultures were isolated from natural substrates, selected by colony isolates of older cultures, or obtained from other laboratories.

General morphological and cultural characteristics of S. griseus. *S. griseus* is characterized by certain morphological and cultural properties that make possible its identification and its ready separation from other species of *Streptomyces.*

Drechsler (1919) made the first comprehensive study of the morphology of *S. griseus.* He reported that the aerial mycelium of the organism showed proliferations of fertile branches at moderately close intervals along the axial hyphae, but no spirals. This early description and the illustrations correspond well with the production of tufts in the aerial mycelium of *S. griseus* found in recent photomicrographs (Carvajal, 1946a, Reynolds and Waksman, 1948). The early description of *S. griseus* (Waksman, 1919) covers the cultural and biochemical properties of the organism, which as a rule characterize rather well both the streptomycin-producing and grisein-producing cultures, with only certain minor differences.

S. griseus produces a typical growth on solid media. On glucose nitrate agar, it forms a thin, spreading growth, developing deep into the medium, at first colorless, then turning olive buff. The pigment of the growth may be lost on successive transfers. The aerial mycelium is abundant, powdery, and of a water-green color. Growth on a potato plug is lichnoid and yellowish or cream-colored; the aerial mycelium is powdery with characteristic greenish pigmentation; the plug remains colorless or tends to become slightly brown. On gelatin, at 18 C, *S. griseus* produces a cream-colored to greenish-yellow growth, with white-gray to greenish aerial mycelium; the gelatin is rapidly liquefied, without the formation of a soluble pigment. Milk is rapidly peptonized with complete clearing, with or without previous coagulation.

The organism is strongly proteolytic and can attack starch, sugars, sugar alcohols, and organic acids. It can obtain its nitrogen from a large variety of compounds, both organic and inorganic.

S. griseus undergoes a typical life cycle. The spores germinate rapidly and produce a typical, monopodially branched mycelium, followed by the formation of a sporulating mycelium. The culture undergoes rapid lysis, especially under submerged conditions of growth, until it is completely disintegrated. The onset of lysis is accompanied by maximum streptomycin production; the completion of lysis is accompanied by a decrease in streptomycin concentration or potency. The glucose-containing media first become slightly acid, then change to alkaline, reaching pH 8.6 to 8.8 during lysis of the culture.

The streptomycin-producing strains of *S. griseus* are subject to destruction by actinophage, which is distinct from that of lysis of the culture, as is brought out

elsewhere (Reilly, Harris, and Waksman, 1947). Phage-resistant strains are produced readily, although these may still carry some of the phage; the streptomycin-producing capacity of such resistant strains does not differ from that of the parent cultures.

Cultures used for comparative studies. Eleven representative cultures of *S. griseus* were taken from the collection for a comparative study of their cultural and biochemical properties. The source and antibiotic-producing capacity of these cultures are shown in table 5. On the basis of their ability to produce antibiotic substances, these cultures can be grouped as follows:

(1) The streptomycin producers included nos. 3463, 3464, 3481, and 3496, the last culture being a colony isolate of the first. These cultures were all sensitive

TABLE 5

Nature and antibiotic activity of cultures studied

COLLECTION NO.	ORIGIN OF CULTURE	ANTIBIOTIC SPECTRUM*	SENSITIVITY BY CROSS-STREAK METHOD† TO		SENSITIVITY TO PHAGE‡
			3463	3478	
3326	1915 culture kept in N. J. collection	0	++++	++++	0
3326a	1915 culture kept in Baarn collection	+	++++	++++	0
3463	Original streptomycin-producing 18-16	±ac	0	+	+
3464	" " " D-1	±ac	+	++	+
3481	Freshly isolated streptomycin-producing culture	±ac	0	+	+
3496	Colony (no. 4) from 3463	±	0	++	+
3478	Grisein-producing, original 25-G	±	++	0	0
3510	Produces a griseinlike substance, 16-F	±	+++	0	0
3527	Produces a griseinlike substance	±	+++	0	0
3495	Pink variant of 3463	+	+++	0	0
3522	Bucherer culture of *S. griseus* obtained from Baarn collection	0	++++	+++	0

* + = active against gram-positive bacteria only; ± = active against both gram-positive and gram-negative bacteria; ±ac = ±, but also active against acid-fast bacteria; 0 = inactive.

† ++++ = very sensitive; +++ and ++ = sensitive; + = slightly sensitive; 0 = not sensitive.

‡ + = sensitive to phage; 0 = resistant to phage.

to actinophage; they were resistant, by the cross-streak method, to the antibiotic effect of the original streptomycin-producing culture 3463 and were also partly sensitive to the effect of the grisein-producing 3478.

(2) The grisein producers included nos. 3478, 3510, and 3527. These were resistant to the action of actinophage; they were sensitive to the antibiotic effect of the streptomycin-producing 3463 and were resistant to the action of the grisein-producing 3478.

(3) Two cultures that produced antibiotics other than streptomycin or grisein were included in this group, namely 3526a, the Baarn culture derived from the original 1915 isolate, and 3495, the pink mutant derived from one of the streptomycin-producing cultures. These two cultures produced antibiotics that had no

effect upon gram-negative bacteria. They were resistant to actinophage. They were sensitive to the antibiotic action of 3463; one was sensitive and the other was resistant to the action of 3478.

(4) The group of inactive cultures included 3326, the original 1915 isolate kept in the New Jersey collection, and 3522, a culture isolated by Bucherer and received from the Baarn collection. The were resistant to actinophage and were very sensitive to both the streptomycin-producing 3463 and the grisein-producing 3478.

Morphologically, none of these cultures could be distinguished from another. They all produced straight aerial mycelium, with a tendency to form tufts, and no spirals. The manner of sporulation, of spore germination, and of growth both in stationary and submerged culture was similar to that reported by Drechsler (1919) and by Carvajal (1946a,b). There were minor variations among the cultures, as in the rapidity of lysis and in the pigmentation of the aerial mycelium; these variations did not appear to be fundamental in nature and could not be associated with the formation of any particular antibiotic.

The cultures were also similar in their characteristic growth upon different media, the variations being more of degree than of kind. Some of the more significant cultural characteristics are listed here.

Growth on gelatin. All the cultures grew well on the gelatin, and nearly all brought about rapid liquefaction of the gelatin. Cultures 3326, 3326a, and 3522 produced only slow liquefaction; this reduction in proteolytic potency may have been the result of the storage of these cultures in collections for many years. It is to be recalled that 3326 was originally one of the strongest proteolytic actinomycetes (Waksman, 1919).

Only the streptomycin-producing strains formed a brownish pigment in gelatin media; this pigment was faint and quite distinct from the type of pigment usually produced by the chromogenic actinomycetes, as, for example, by *Streptomyces lavendulae* or *Streptomyces scabies.* Of the other cultures, 3495, the pink mutant, was the only one to produce also a slight pigmentation of the gelatin.

Potato plug. All cultures produced a lichnoid, somewhat brownish growth on potato plugs. It rapidly became covered with a white, powdery aerial mycelium. The greenish tinge of this mycelium was more pronounced in the case of the streptomycin-producing cultures; the pigmentation of the mycelium either remained white or tended to become gray in the case of the other cultures. The color of the plug was unchanged, except in the case of the streptomycin-producing strains, which tended to produce a faint brownish pigment around the growth.

Nutrient agar. Growth was similar for all cultures, being cream-colored and lichnoid in appearance. The aerial mycelium was powdery, white to light gray. The only differences observed were in the amount of mycelium covering the growth. None of the cultures produced any soluble pigment.

Glycerol agar. With the exception of the pink variant 3495, the color of which was carmine red, all the cultures produced a thin, cream-colored growth with white to gray aerial mycelium. Only the streptomycin-producing strains and the pink variant produced the typical greenish pigmentation of the mycelium.

Glucose asparagine agar. On this medium as well, all the cultures, with the

exception of 3495, which produced a vinaceous-colored growth, produced a thin, cream-colored growth. The aerial mycelium was light gray, varying in abundance with each culture. The greenish tinge of the streptomycin-producing cultures and the mouse-gray color of 3522 were the exceptions.

Starch agar and Czapek's agar. Cream-colored growth, similar for all cultures except for 3495, in which it was somewhat pinkish to lavender, was produced. Aerial mycelium of most of the non-streptomycin-producing strains was white to cream-colored, varying in abundance and in shade of color; 3522 produced a mouse-gray mycelium; the streptomycin-producing strains, as well as 3495, were covered with a greenish mycelium.

These results show that it is hardly possible to separate any one group from the others in an attempt to create a separate species. It could possibly be done for the streptomycin-producing group or for the pink variant. The streptomycin cultures are characterized by their ability to produce streptomycin, their sensitivity to actinophage, the greenish pigmentation of the aerial mycelium, and the slight brownish pigment on gelatin and certain other complex organic media. The fact, however, that 3326 and 3326a when originally isolated also produced the typical watery greenish pigmentation of the mycelium, and also the fact that the grisein-producing cultures frequently show the greenish coloration of the aerial mycelium, would speak against using this property as a major distinguishing characteristic between constituent groups within the species *S. griseus.* The variations in other properties also are not sufficient to justify, for the time being at least, subdividing this species.

SUMMARY

Streptomyces griseus (Krainsky emend. Waksman and Curtis) Waksman and Henrici represents an extremely variable group of organisms.

Only some of the cultures of *S. griseus* isolated from natural substrates are able to produce antibiotic substances. A few cultures produce streptomycin, and a few form other antibiotics.

On the basis of their ability to produce antibiotic substances, the various cultures of *S. griseus* have been grouped into four categories: (1) those that produce streptomycin; (2) those that produce grisein; (3) those that form an antibiotic that is neither streptomycin nor grisein; and (4) those that do not form any antibiotic.

Streptomycin-producing cultures give rise to a number of variants or mutants, which differ in their capacity to form streptomycin quantitatively under the same conditions or under different conditions of cultivation. Some of the variants do not form any streptomycin; others may form a different type of antibiotic.

A study of the morphological characteristics and cultural properties of different strains of *S. griseus* revealed sufficient similarity between them to justify considering them all, for the time being at least, as members of a single, very variable species.

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THE RESPIRATION OF SALMONELLA IN THE PRESENCE OF AGGLUTINATING SERUM[1]

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The literature concerning the effect of immune serum upon the activities of microorganisms has been fully reviewed and discussed by Sevag (1945). Sevag and Miller (1948) recently reported that the agglutination of typhoid organisms does not inhibit oxygen uptake by the cells, whereas lysed cells show an immediate increased rate of oxygen consumption followed by a marked decrease. The work herein reported consisted of a series of experiments to determine whether combination of agglutinins with the cells of a number of *Salmonella* species had any effect on their respiratory activity.

Cells for studies were obtained by culturing the organism on glycerol thiosulfate agar. Sufficient growth occurred in 24 hours to yield a heavy suspension of cells suitable for use either as antigens or for enzyme studies. Cells were suspended in a balanced salts-buffer solution (Harris and Gainey, 1944) and washed twice by centrifuging, after which they were suspended in a concentration of approximately 100 times the turbidity of MacFarland nephelometer tube no. 1, and cooled to 3 C.

Normal adult rabbits were immunized by a series of intravenous injections of heat-killed cultures. The immune serum was obtained by centrifugation following clotting of blood removed by cardio-puncture. All tests were conducted the same day the rabbits were bled. Agglutination tests to determine the antibody titer followed the usual procedure for tube tests.

Oxygen utilization was measured in the Warburg microrespirometer at 37 C, following the usual technique of Dixon (1934). Twentieth molar glucose, buffered at pH 7.0 with phosphate salts, was used as the respiration substrate in all experiments. Cells grown and harvested in the manner described were quite active physiologically and exhibited little or no endogenous respiration. The methylene blue reduction tests followed the Thunberg technique as described by Quastel and Whetham (1925). Visual judgment of the complete reduction of the dye was taken as the end point. Although this method is subject to error, significant differences in the rate of reduction of the methylene blue can be observed.

In measuring oxygen uptake by microorganisms with the manometric method, the formation of hydrogen gas by the cells may lead to significant errors. However, in no instance in this investigation was a positive gaseous pressure recorded during an experimental period of 2½ hours when nitrogen replaced the air in the respiration flasks and alkali was present to absorb carbon dixoide. Hence it may be assumed that no appreciable volume of hydrogen was liberated by the

cells under the experimental conditions. This failure to liberate hydrogen was probably due to the organism's inability to form the necessary adaptive enzyme, hydrogenlyase, following growth on the glucose-free glycerol thiosulfate medium (Stephenson and Stickland, 1933). The experiments were of such short duration that no appreciable subsequent growth and enzyme formation could be expected to take place in the respirometer.

When either normal or immune serum was added to the bacterial cells, buffer, glucose system in the respirometer, an appreciable increase in the rate of oxygen uptake was observed as compared to that found when glucose alone was used. Warren (1945) found that addition of immune serum to luminescent bacteria gave increased light production. He attributed this increased activity to the

TABLE 1

Average oxygen uptake by Salmonella species on glucose in the presence of normal and immune rabbit serum

SPECIES STUDIED	CELLS PER FLASK	O_2 UPTAKE IN MM^3 PER HOUR IN THE PRESENCE OF RABBIT SERUM*						
		Normal	Immune					
		0.1 ml	0.1 ml	0.05	0.025	0.01	0.005	0.001
	billions							
S. schottmuelleri..	1.45	78	*62*	*72*	*70*	*76*	*76*	*75*
S. paratyphi	1.67	134	*136*	*153*	*131*	*119*	*109*	116
S. california	1.67	105	*95*	*103*	*103*	*107*	*107*	104
S. stanley	1.43	139	*136*	*137*	*138*	*140*	*143*	138
S. derby	1.53	115	*111*	*114*	*114*	*116*	*108*	109
S. typhimurium.	2.03	107	*105*	*118*	*114*	*120*	109	110
S. gallinarum	1.27	45	*44*	*41*	*40*	39	39	39
S. pullorum.	1.60	70	*78*	*69*	*71*			*73*

* Normal rabbit serum added to give total volume of serum equal to 0.1 ml; values italicized represent flasks with sufficient agglutinin to give complete agglutination.

utilization of metabolites present in the serum. In order to eliminate variations due to such differences, comparisons were made of oxygen uptake of *Salmonella* in normal and immune sera and in mixtures of these. When the quantity of immune serum was less than 0.1 ml, normal serum was added to bring the total volume of serum to 0.1 ml. In this way the quantities of metabolites and the quantity of the natural complement added to the respirometer flasks were held fairly constant.

DISCUSSION OF RESULTS

The cubic millimeters of oxygen utilized in 1 hour by eight species of *Salmonella* in the presence of varying quantities of immune and normal rabbit serum are shown in table 1. The italicized values indicate experiments in which sufficient agglutinin was present for complete agglutination of the cells. The agglutination titers of the sera varied from relatively low titer in the case of *Salmonella gallinarum* to relatively high titer for *Salmonella schottmuelleri*. The quantities

of agglutinin added to the respiration flasks ranged from a concentration which gave no visible clumping of the cells to 500 times the quantity necessary for complete agglutination.

The quantities of oxygen utilized in normal and in immune serum show slight variations, but there was no consistent decrease in respiratory activity that could be attributed to antibodies. The recorded differences might be due to slight variations in the quantity of available metabolites in the sera. Varying the ratio of antibody to antigen did not give a zone of optimal inhibition of respiration.

In the experiments just described, the rabbits were immunized with heat-killed bacterial cells, which would give rise only to antibodies against the somatic antigens of the dead organisms. In order to determine whether living bacteria, with and without flagellar antigens, might produce different results, rabbits were immunized first with killed cultures and then were subjected to inoculations with live broth cultures. In other experiments, adult hens were given intra-peritoneal inoculations of live pullorum organisms. Respiration studies using the antisera from these animals gave results similar to those shown in table 1.

The results obtained in these experiments agree with those reported by Sevag and Miller (1948) for the intact sensitized typhoid organisms. None of the strains used exhibited marked bacteriolysis in the presence of the fresh immune serum. In several instances the complement concentration was increased by the addition of fresh guinea pig serum without affecting the rate of oxygen utilization or causing lysis of the sensitized cells.

Preliminary studies of dehydrogenation of various organic substrates by *Salmonella* species in the presence of blood serum indicated that normally the quantity of metabolizable compounds in the serum alone was sufficient to allow rapid reduction of the methylene blue. To avoid this complication, cell suspensions were mixed with an equal quantity of fresh normal or immune serum and incubated at 37 C for 3 hours. The cells were then removed by centrifuging and were washed twice in the balanced salt solution. Cells from the immune serum were still combined with enough agglutinin to agglutinate on standing a few minutes, but neither those from normal nor immune serum reduced methylene blue more rapidly in the absence of added substrate than did untreated control cells.

Methylene blue reduction time data in the presence of 0.1 molar glucose, sodium lactate, and sodium formate are recorded in table 2. These three compounds were rapidly oxidized by all the species studied. Reduction was almost as rapid in the presence of glycerol and mannitol as in the presence of glucose, but sodium succinate, sodium gluconate, ethyl alcohol, and sodium acetate were utilized only slightly or not at all. Treatment of cells with fresh immune serum gave no evidence of inhibition of enzymic activity by antibody in the presence of any substrate studied.

Measurement of both oxygen uptake and the dehydrogenase activity of intact cells has demonstrated no significant differences between the effect of normal and immune serum on the respiratory activity of the salmonellae. Apparently,

combination of the specific agglutinins with the bacterial cell and their subsequent agglutination have no direct effect upon enzymes responsible for the activation of the organic substrate. Also, agglutination apparently does not interfere with the mechanism for transporting electrons between the substrate-activating portion of the respiratory system and that part combining with molecular oxygen.

TABLE 2

Time in minutes required for the reduction of methylene blue by Salmonella species treated with normal and immune serum

SPECIES	CELLS TREATED WITH NORMAL SERUM	CELLS TREATED WITH IMMUNE SERUM
Sodium lactate		
	min	min
S. pullorum	15	15
S. paratyphi.	15	15
S. stanley	22	20
S. typhimurium.....	60	65
Glucose		
S. pullorum	25	25
S. paratyphi.........	15	15
S. stanley	20	20
S. typhimurium	65	65
Sodium formate		
S. pullorum	40	40
S. paratyphi.	40	45
S. stanley	15	15
S. typhimurium.........................	35	30

SUMMARY

Oxygen uptake, with glucose as the substrate, by eight species of *Salmonella* occurred at essentially the same rate when the cells were in the presence of fresh immune rabbit serum as when in fresh normal serum. Varying the proportions of agglutinin to cells over a wide range, with sera of both high and low titer, gave no indication of a zone exhibiting antienzyme activity affecting respiration. Dehydrogenase activity of four species was studied upon nine organic substrates. Reduction of methylene blue in the presence of a specific substrate occurred at the same rate when the cells were treated with fresh immune serum as with normal agglutinin-free serum.

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THE EFFECT OF pH UPON THE BACTERIOSTATIC ACTIVITY OF CERTAIN NITROPHENOLS[1]

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Although it is generally realized that the bacteriostatic activities of acidic substances as a rule increase as the pH of the culture medium is lowered, there appears to be in the literature no information on the rates at which such changes occur in terms of the concentrations and the ionization constants of the substances and the pH of the medium. In this paper are presented some data on the bacteriostatic powers of a number of nitrophenols for which the inhibiting concentrations at different culture pH values were determined.

As test organisms cultures of *Escherichia coli* and *Bacillus mesentericus* were used, and for media both extract broth and simple chemically defined media were employed. Since the results with the two species and the two media were essentially the same, only the data obtained with *E. coli* grown in extract broth will be given.

The broth and the broth solutions of the compounds were adjusted with HCl or NaOH to pH values of 5.5, 6.5, 7.5, and 8.5. The concentrations of the substances under test were, as a rule, decreased in 3:4 ratio by transferring 15-ml volumes through tubes containing 5 ml of broth. This gives a logarithmic reduction in concentration to $\frac{1}{10}$ of the original value through a series of nine tubes. The inoculum consisted of one drop of a 1:1,000 dilution of an 18-hour broth culture, and incubation was at 37 C.

The bacteriostatic value of a substance was taken as the lowest molar concentration preventing visible growth for 4 days, and the final value used was the geometric mean of several determinations, usually four or five. These figures are included in the following table of the compounds with their pKa constants.

An examination of the graph, from which the curves of several of the compounds have been omitted for the sake of simplicity, shows that, in general, the more acid a substance is, the greater is the effect of pH change upon its bacteriostatic power. *m*-Nitrophenol, with a pKa of 8.3, is but little affected throughout the range of culture pH studied, whereas a compound such as 2,5-dinitrophenol, pKa 5.1, increases rapidly in potency as the pH falls. The behavior of picric acid may be due in part to the fact that it is present almost exclusively in the ionic form, and, as a rule, cell membranes are relatively impermeable to such anions. The irregular action of picric acid is duplicated at a high pH (8.5) by the other more acidic compounds.

The behavior of the nitrophenols contrasts markedly with that of the large series of acridine derivatives studied by Albert and his colleagues (1945). The

latter substances seem to owe their activity to the cation, so that the most basic and hence most highly dissociated compounds are the most effective bacteriostatic agents. The activity is directly proportional to the concentration of cations existing at the culture pH values studied and is apparently due to com-

TABLE 1

	pK_a	MOLAR CONCENTRATIONS INHIBITIVE AT pH VALUES OF			
		5.5	6.5	7.5	8.5
m-Nitrophenol	8.3	.0028	.0026	.0028	.005
p-Nitrophenol	7.1	.0007	.001	.0025	.0044
2-Amino,4-nitrophenol	7.0	.002	.0033	.008	.025
2,5-Dinitrophenol	5.1	.00011	.00035	.0011	.0044
2-Amino,4,6-dinitrophenol	4.4	.00056	.003	.011	.02
2,4-Dinitrophenol	4.0	.00028	.0011	.0056	.016
2,6-Dinitrophenol	3.6	.00032	.0016	.011	.018
2,4,6-Trinitrophenol	0.8	.0035	.009	.014	.014
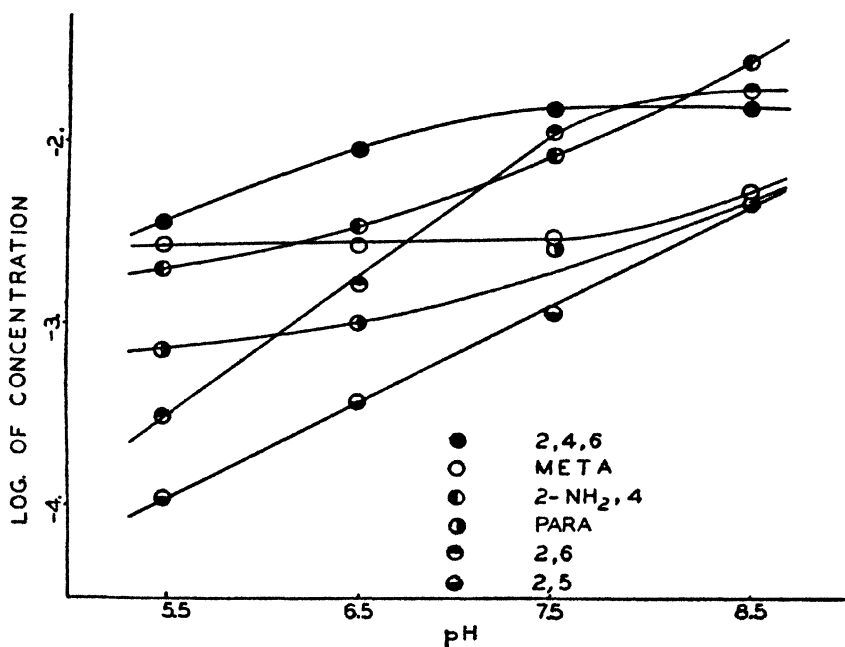
Figure 1. Bacteriostatic action of nitrophenols.

petition of these with hydrogen ions for some position on an enzyme surface. Since the oil-water partition coefficients of the active acridines are close to unity, it is deduced that the bacteriostatic action takes place in the aqueous phase, probably at the cell surface.

With the nitrophenols, on the other hand, there is no apparent relationship

between the concentrations of the anions of the bacteriostatic substances and the concentration of hydroxyl ions. This, together with the decrease in efficacy as the anion: molecule ratio increases, suggests that the substances owe their activity to the undissociated compound.

In an effort to explain this behavior of the nitrophenols by established chemical principles, the data have been examined from the standpoint of the law of mass action. An approach similar to that used previously (Klotz, 1944) in an analysis of sulfonamide action has yielded expressions that are in semiquantitative agreement with the observations recorded in this paper.

For the simplest treatment it has been assumed that the phenol is active in its nonionized form, that no significant fraction of it is removed by the bacterial enzymes or other proteins, and that the effect of pH on these proteins may be neglected. The first assumption is supported by the results reported in this paper and by the work of Krahl and Clowes (1938), who found, with similar substituted phenols, that the inhibition of cell division in the case of *Arbacia punctulata* eggs is dependent on the concentration of the phenol molecules in the external medium, and hence in the cell, and is independent of the concentration of the anions. The second assumption is, of course, quite arbitrary and is made only as a first approximation to simplify the theoretical treatment. This assumption is eliminated in the second treatment, which is described below. The third assumption is based on a picture of binding occurring by a hydrogen bridge between the nonionized phenol molecule and an anionic carboxylate group on the protein. In the region under investigation, pH 5.5 to 8.5, the carboxyl groups are completely ionized and not subject to modification by changes in acidity.

For the purposes of the following derivation we may set up these definitions:

HD = nonionized phenol
D⁻ = ionized phenol
C = HD + D⁻ = total phenol required to produce bacteriostatis.

From the law of mass action one may write the equilibrium expression

$$\frac{(H^+)(D^-)}{(HD)} = K \tag{1}$$

where K is the ionization constant of the drug. It follows immediately with the aid of equation (1) that

$$C = \frac{(H^+)(D^-)}{K} + (D^-) \tag{2}$$

It is desired to know the variation of C with (H^+) under conditions such that (HD) is kept constant and bacteriostasis is maintained. For this purpose one may differentiate equation (2) with respect to (H^+) to obtain:

$$\frac{dC}{d(H^+)} = \frac{H^+}{K}\frac{d(D^-)}{d(H^+)} + \frac{(D^-)}{K} + \frac{d(D^-)}{d(H^+)} \tag{3}$$

Since

$$(D^-) = C - (HD) \tag{4}$$

then

$$\frac{d(D^-)}{d(H^+)} = \frac{dC}{d(H^+)} \tag{5}$$

since (HD) is constant. Hence equation (3) becomes

$$0 = (H^+) \frac{dC}{d(H^+)} + (D^-) \tag{6}$$

Consequently, one may obtain the expression

$$\frac{(H^+)}{C} \frac{dC}{d(H^+)} = \frac{d\ln C}{d\ln(H^+)} = -\frac{(D^-)}{C} = -\frac{(D^-)}{(HD) + (D^-)} = \frac{K}{K + (H^+)} \tag{7}$$

Therefore,

$$\frac{d \log C}{d \, ph} = \frac{K}{K + (H^+)} \tag{8}$$

From equation (8) it is evident that when the drug is a very weak acid, (H^+) in the culture range is always greater than K and the slope of the log C–pH line will be a small number approaching zero. This is the observed behavior with *m*-nitrophenol (pK 8.3) until pH 8.5, at which (H^+) and K are comparable in size and the slope should, and does, increase. For such compounds as *p*-nitrophenol (pK 7.1) and 2-amino-4-nitrophenol (pK 7.0) where the pK values fall in the culture pH range, the slope should tend to rise as the culture pH rises. This is seen to be the case. With the very acid compounds, (H^+) in the denominator of equation (8) is negligible and hence the slope should be 1 and independent of pH in the culture range. For 2,5-dinitrophenol (pK 5.1) and 2,6-dinitrophenol (pK 3.6) the slopes are substantially constant except perhaps at high pH for the latter drug. On the other hand, the experimental observations definitely depart from theory in that the slopes for these very acid phenols are not unity, though they do tend to increase toward 1 as the acidity of the drug increases. The largest slope found in this study is 0.75, with 2,6-dinitrophenol.

The basis of this discrepancy probably lies in the assumption that none of the phenol is removed by the bacteria. It has seemed appropriate, therefore, to develop an expression for the slope of the log C–pH plot which takes into account the possibility that a significant portion of the nonionized form may be bound by the bacteria. For this purpose we must revise our definition of C to

$$C = HD + D^- + PHD$$

and we define T, the total protein present, by the expression

$$T = P + PHD$$

Since we are now considering two equilibria, we must set up two equilibrium constants

$$\frac{(H^+)(D^-)}{(HD)} = K \tag{1}$$

$$\frac{(HD)(P)}{(PHD)} = k \tag{9}$$

With these definitions and equilibrium expressions, one can derive the following equation for the slope, by a procedure analogous to that described above, if we assume that bacteriostasis is maintained by keeping constant values of bound phenol, PHD.

$$\left(\frac{d \log C}{d pH}\right)_{PHD\ constant} = \frac{K}{K + (H^+)\left[1 + \dfrac{P}{k}\right]} \tag{10}$$

Since this expression contains two parameters, P and k, which are unknown, it is not possible to predict explicitly the value of the second term in the denominator. Nevertheless, it is obvious that the additional factor $(1 + P/k)$, would tend to increase the magnitude of the denominator and hence make the slope less than 1 even for very acid compounds. It is thus evident that this more detailed analysis gives better agreement with the experimental observations.

Consideration has been given also to the possibility that the phenol may not be bound to carboxylate ions but rather to a basic nitrogen atom on the protein. In such a situation the assumption of pH independence on the part of the protein might be erroneous. If one wishes to take pH effects into account, it is necessary to introduce a third equilibrium constant, k_3, to correlate the acid properties of the protein. It is convenient to define k_3, and the auxiliary constants K' and k' in the following manner:

$$\frac{(HP^+)}{(H^+)(P)} = k_3 \tag{11}$$

$$\frac{(HD)}{(H^+)(D^-)} = K' = \frac{1}{K} \tag{12}$$

$$\frac{(PHD)}{(P)(HD)} = k' = \frac{1}{k} \tag{13}$$

By differentiation and algebraic manipulation similar to that described already one obtains a more detailed expression for the slope of the log C — pH curve

$$\frac{d \log C}{d pH} = \frac{1 - K'k_3(H^+)^2}{[1 + k_3(H^+)][1 + K'(H^+)] + K'k'(H^+)[T - (PHD)]} \tag{14}$$

It is apparent, however, that this relationship is quite unwieldy and hence of little immediate value in connection with the present problem.

CONCLUSIONS

For most members of a group of nitrophenols examined, the changes in bacteriostatic potency at different culture pH levels can be correlated with the acid strengths of the compounds. A weakly acidic substance (pKa between 8 and 9) is inhibitive in almost the same concentration throughout a culture pH range of 5.5 to 8.5, whereas a more acid compound (pKa in the neighborhood of 4, for example) may undergo a 60-fold change in potency through the same range. The acitivity seems to be due to the undissociated molecule of the drug.

By a mass law approach a simple formula may be derived that gives semi-quantitative agreement with the experimental findings. Two other formulae are offered, which would in all probability agree much more closely with these facts, were the proper equilibrium constants available.

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THE PRODUCTION, ASSAY, AND ANTIBIOTIC ACTIVITY OF ACTIDIONE, AN ANTIBIOTIC FROM STREPTOMYCES GRISEUS

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Actidione is a chloroform- and water-soluble antibiotic that is produced by streptomycin-yielding cultures of *Streptomyces griseus* and that, unlike streptomycin, possesses no marked antibacterial activity (Whiffen, Bohonos, Emerson, 1946). It is, however, highly active against a large number of yeasts, including the pathogen *Cryptococcus neoformans*. Actidione has been obtained in pure crystalline form (Leach, Ford, and Whiffen, 1947) and its empirical formula has been determined (Leach and Ford, 1948).

The purpose of this paper is to report studies on the production, assay, and antibiotic activity of actidione. The production by the same organism of two easily differentiated antibiotics has offered an opportunity for the study of several problems of theoretical interest. It has been possible to determine the effect of changes in the composition of the fermentation medium upon the relative yields of actidione and streptomycin and to demonstrate that the amounts of the two antibiotics produced may vary independently of one another. It was of further interest to determine whether or not the loss by mutation of the ability to synthesize one antibiotic would result in an inability to synthesize the other antibiotic.

PRODUCTION OF ACTIDIONE

Effect of composition of medium upon relative yields of streptomycin and actidione. Actidione was produced in 100-ml aliquots of medium contained in 500-ml Erlenmeyer flasks that were incubated in a reciprocal shaker at 24 C. The flasks were inoculated with 5 ml each of a 72-hour-old vegetative growth of *S. griseus*, phage-resistant strain RM-241CR (Saudek and Colingsworth, 1947). Assays for actidione were performed by a paper-disk plate method that is to be described later. Streptomycin was assayed by the method of Loo *et al.* (1945).

The yields of actidione and streptomycin were first compared in two different media, identified as no. 25 and no. 26. Medium 25 contained the following: "cerelose" 20.0 g, KCl 4.0 g, $CaCO_3$ 8.0 g, $(NH_4)_2SO_4$ 5.0 g, dried brewers' yeast (Standard Brands no. 2019) 2.5 g, KH_2PO_4 0.2 g, distilled water 1,000 ml. In this medium high yields of streptomycin were obtained, but only small amounts of actidione were produced (table 1). On the other hand, the yield of actidione was superior to that of streptomycin in medium 26, whose composition was as follows: cerelose 10.0 g, soybean oil meal 10.0 g, "curbay B-G" (a distiller's soluble from U. S. Industrial Alcohol) 5.0 g, $CaCO_3$ 1.0 g, NaCl 5.0 g, distilled water 1,000 ml (table 1).

As will be noted, medium 25 is a mixture of inorganic salts to which glucose and yeast are added as organic supplements. Of a number of organic substances tried as a substitute for yeast in medium 25, none, with the possible exception of

TABLE 1

Relative yields of actidione and streptomycin in three different media

TRIAL NO.	MEDIUM 25		MEDIUM 32		MEDIUM 26	
	Actidione	Streptomycin	Actidione	Streptomycin	Actidione	Streptomycin
	ug/ml	*µg/ml*	*ug/ml*	*µg/ml*	*µg/ml*	*µg/ml*
1	60	320	134	344	134	160
2	66	220	69	212	122	136
3	61	280	24	148	124	116
4	50	240	210	344	276	80
5	45	272	117	212	300	260
6	10	164	158	240	284	190
7	52	82	160	264	384	208
Mean	49	225	124	252	232	164

TABLE 2

Effect of various substituents for yeast in medium 25 upon the yields of actidione and streptomycin

g	SUBSTITUENT FOR YEAST IN MEDIUM 25	ACTIDIONE	STREPTOMYCIN
		µg/ml	*µg/ml*
2.5	Soybean meal	27	68
2.5	Soybean meal	31	90
2.5	Wilson's solubilized liver	47	240
2.5	GBI vitamin test casein	4	10
2.5	N-Z amine	2	3
3.0	Peptone (Difco)	8	13
3.0	Beef extract (Difco)	9	28
3.0	Tryptone (Difco)	9	17
1.0	GBI casein hydrolyzate	56	33
5.0	Brewers' yeast	56	300
1.0	Curbay B-G	<2	17
2.5	Curbay B-G	136	204
2.5	Curbay B-G	101	160
5.0	Curbay B-G	186	208
10.0	Curbay B-G	170	108
20.0	Curbay B-G	75	66
	Yeast omitted	<2	7

solubilized liver, was as effective as yeast in promoting the production of streptomycin, and only curbay B-G was stimulatory to the formation of actidione (table 2). An increase in concentration of curbay B-G from 1 gram to 5 grams per liter increased the yield of actidione from less than 2 micrograms per ml to 186 micrograms per ml. A concentration of 10 g of curbay B-G per liter was

inhibitory to streptomycin production and had no further stimulatory effect on actidione. A still higher concentration of 20 g of curbay B-G per liter was inhibitory to the production of both actidione and streptomycin. Furthermore, when 2.5 g of curbay B-G per liter are added to medium 25 (medium 32), the result is a 100 per cent increase in the yield of actidione over that obtained in unmodified medium 25 (see medium 32 in table 1).

The effect of mutation upon the relative yields of actidione and streptomycin. As has been shown, the relative yields of actidione and streptomycin that may be obtained from any one strain of *S. griseus* are dependent upon the composition of the fermentation medium. It was also desirable to determine the relative yields of actidione and streptomycin from a large number of different strains of *S. griseus* when all were grown on the same medium. This study was carried out with the co-operation of Dr. George M. Savage of this laboratory, who was

TABLE 3

Distribution of yields of streptomycin and actidione among 144 X-rayed isolations of S. griseus RM-241 when grown on medium 26A

	NUMBER OF ISOLATIONS	PERCENTAGE OF TOTAL ISOLATIONS
Yields of actidione and streptomycin equivalent to that of RM-241.	122	86
Yields of actidione and streptomycin less than 50% of that of RM-241.	7	5
Yield of streptomycin average but yield of actidione less than 50% of average for RM-241.	11	7
Yield of actidione average but yield of streptomycin less than 50% of average for RM-241.	3	2

routinely X-raying conidia of *S. griseus* in order to obtain superior streptomycin-producing mutants.

Conidial suspensions of *S. griseus*, strain RM-241, were exposed to X-rays and streaked out on agar plates. The isolations made were cultured in shaker flasks containing 100 ml each of a medium that differed from no. 26 in containing 0.5 g of curbay B-G instead of 5.0 g per liter and is designated as medium no. 26A. In this medium the mean for streptomycin was 250 micrograms per ml, and the mean for actidione was 230 micrograms per ml. Culture filtrates were assayed for both streptomycin and actidione. A total of 144 isolations were tested in this way, and the data thus obtained are to be found tabulated in table 3. Eighty-six per cent of the X-rayed strains produced amounts of actidione and streptomycin that were greater than one-half of the mean for the parent strain. In approximately 5 per cent of the isolations the yields of both actidione and streptomycin were reduced to less than one-half of their means for RM-241. In 7 per cent of the strains the amount of streptomycin produced was greater than one-half the mean for the parent strain, but the amount of actidione was less than one-half of its mean. In only 2 per cent of the strains was the yield of

actidione equal to that of the parent strain and the yield of streptomycin less than the mean for the parent strain. Four conditions prevailed among the 144 isolations: (1) the yields of actidione and streptomycin were within the normal range of variation of the parent strain; (2) the yields of both antibiotics were reduced by at least 50 per cent; (3) the yield of streptomycin was normal but actidione was less than 50 per cent normal; and (4) the yield of actidione was normal but streptomycin was less than 50 per cent normal.

In a further effort to discover the existence of strains in which there had occurred a loss in ability to synthesize one antibiotic without an accompanying effect upon the yield of the other antibiotic, there were selected for further study 38 strains in which the yield of streptomycin was less than 2 micrograms per ml and 31 strains in which the yield of actidione did not exceed 20 micrograms per ml. These 69 strains were grown in shaker flasks on medium 25A and assayed for streptomycin and actidione. Of the 38 strains that were inactive in respect to streptomycin, 25 produced no actidione, and 13 produced less than 50 micrograms of actidione per ml. None of these strains were normal in respect to the yield of actidione. However, among the 31 strains producing less than 20 micrograms of actidione per ml there were 8 in which the yield of streptomycin was greater than 300 micrograms per ml and 5 that produced less than 2 micrograms of streptomycin per ml. The yields of streptomycin from the remaining 18 strains were less than the mean of that antibiotic for the parent strain on medium 26A. It is possible, therefore, for a loss to occur in the ability to synthesize one antibiotic without an effect upon the production of the other antibiotic. The more frequent condition, however, under the experimental conditions employed, was one in which the ability to produce both antibiotics was impaired.

ASSAY OF ACTIDIONE

The paper-disk plate method of streptomycin assay (Loo *et al.*, 1945) was adapted to the assay of actidione. The test organism is *Saccharomyces pastorianus* ATCC 2366, which is unaffected by concentrations of streptomycin as high as 1 mg per ml. The assay medium is as follows: glucose 10 g, Difco yeast extract 2.5 g, KH_2PO_4 1.0 g, agar 20 g, and distilled water 1,000 ml; pH 6.0. The assay standard is prepared with crystalline actidione in 0.1 M PO_4 buffer at pH 5.0. The following concentrations of actidione are used to obtain the standard curve: 6.0, 4.0, 2.5, and 1.5 micrograms per ml.

A study was made of some of the factors affecting the reproducibility of the actidione assay. These factors include hydrogen ion concentration, the presence of various electrolytes and organic diluents in the assay samples, and the rate of inoculation of the assay medium.

The most important single factor to be controlled in the assay of actidione is the rate of inoculation of the assay medium. Increasing concentrations of actidione are required to inhibit increasing concentrations of yeast cells. A decrease in the concentration of yeast cells results in an increase in the diameter of the zone of inhibition. This marked inoculum size effect is demonstrated in figure 1. Each 10-fold increase in the concentration of inoculum is accompanied

by a 25 to 50 per cent apparent decrease in the sensitivity of the assay, depending upon the portion of the assay curve involved.

To determine the possible effect of pH upon the size of the zone of inhibition produced by actidione, dilutions of actidione were made in 0.1 M PO_4 buffer at various pH's to give final actidione concentrations of 10.0, 5.0, 2.5, and 1.25 micrograms per ml. The range of pH was as follows: 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 7.5, and 7.9. Variations in the pH of the actidione solutions had no measurable effect upon the assay. Likewise when the pH of aliquots of agar assay medium was adjusted to cover a range of 4.0 to 8.0, there resulted no detectable difference in the diameters of the zones of inhibition produced by the actidione standard.

Since the presence of electrolytes in solutions to be assayed has been reported as having marked effect on the assay of streptomycin (Loo *et al.*, 1945), it seemed
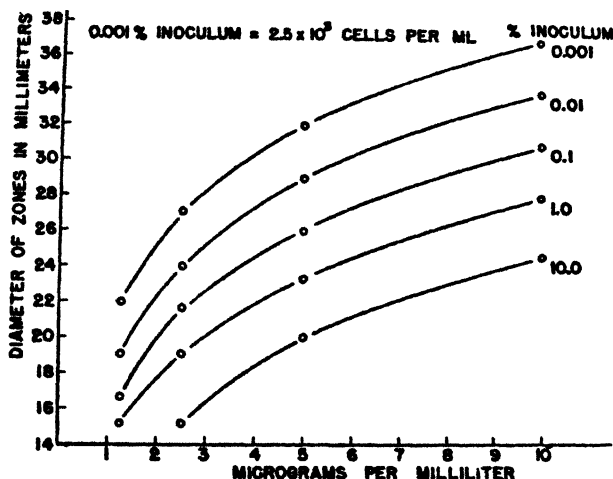
Figure 1. Effect of concentration of inoculum upon the size of the zone of inhibition of *S. pastorianus* by actidione.

advisable to determine whether or not the assay of actidione would be influenced by salts in the assay solutions. Solutions of 0.1, 0.01, and 0.001 molarity were prepared of the following salts: KCl, $MgSO_4$, $FeSO_4$, $ZnSO_4$, NaCl, $CuSO_4$, and $K_2HPO_4 + KH_2PO_4$. Actidione was diluted in the various salt solutions to concentrations of 10.0, 5.0, 2.5, and 1.25 micrograms per ml. Salt solutions without antibiotic and antibiotic solutions without salts were run as controls. At concentrations of 0.01 M and 0.001 M the salts had no effect upon the size of the zone of inhibition. At 0.1 M $CuSO_4$ exerted a marked toxic effect upon the test organism that resulted in enlargement of the zone of inhibition. To a lesser extent $MnSO_4$ and $ZnSO_4$ were inhibitory at 0.1 M concentration, but the remaining salts, KCl, $MgSO_4$, $FeSO_4$, and NaCl, were nontoxic and without effect upon the assay at this concentration.

Of six organic solvents tested for inhibitory effect against *S. pastorianus* in 100 per cent concentration only amyl acetate was toxic. At a 50 per cent con-

centration amyl acetate was not inhibitory. The other five solvents, methanol, ethyl acetate, acetone, ethanol, and tertiary butanol, were nontoxic.

The antibiotic action of actidione against S. pastorianus was not antagonized in vitro by beef serum nor by the whole blood or serum of the rabbit.

RESISTANCE OF S. PASTORIANUS TO ACTIDIONE

Preliminary to the development of the assay for actidione, the stock culture of Saccharomyces pastorianus was plated out and 88 colonies picked. Cell suspensions were prepared from each isolation and diluted to equal turbidity to serve as inocula. The amount of actidione required to inhibit completely the growth of each strain was determined by tube dilution assay. The results are tabulated in table 4. Fifty-five per cent of the strains were inhibited by 0.25 microgram

TABLE 4

Sensitivity of 88 strains of S. pastorianus to the antibiotic action of actidione

CONCENTRATION OF ACTIDIONE	NUMBER OF STRAINS COMPLETELY INHIBITED	PERCENTAGE OF TOTAL
μg/ml		
0.5	24	27.4
0.25	49	55.8
0.125	13	14.4
0.09	2	2.4
0.06	0	0.0
Total	88	100.0

of actidione per ml. Only two of the 88 strains were inhibited by less than 0.1 microgram per ml, and one of these was selected for the assay of actidione.

Since frequent subculture of S. pastorianus was inadvisable because of the possible appearance of resistant cells, large quantities of cells were grown simultaneously in shaker flasks. The cell suspensions thus obtained were diluted to desired concentration in PO_4 buffer and refrigerated. The culture could be stored in this way for 3 or 4 months without noticeable deterioration in the viability of the cells.

S. pastorianus developed resistance to actidione rather readily. Beginning with a strain sensitive to 0.06 microgram of actidione per ml, by successive transfers to increasing concentrations of actidione, a strain was obtained on the third transfer that was resistant to 4.0 micrograms per ml. This represented a 16-fold increase in resistance in three transfers.

ANTIBIOTIC SPECTRUM OF ACTIDIONE

The range of antibiotic activity of actidione was determined, using crystalline material, for 22 yeasts, 14 fungal pathogens of man, and 12 bacteria. The spectrum previously published (Whiffen, Bohonos, and Emerson, 1946) had been determined with an impure preparation of actidione. Antibiotic potencies

against yeasts and bacteria were determined by tube dilution and against the pathogenic fungi by an agar streak plate method. Actidione, to give final concentrations up to 1 mg per ml, was added to tubes containing 4 ml of the following medium: Difco peptone 0.75 per cent and Difco yeast extract 0.25 per cent; pH 7.2. These tubes were inoculated with bacteria and incubated 24 hours at 30 C. Actidione was also added to tubes containing 10 ml each of the following medium: glucose 1.0 per cent, Difco yeast extract 0.25 per cent, and K_2HPO_4 0.1 per cent at pH 6.0. These tubes were inoculated from 24-hour-old yeast cultures and incubated at 30 C for 18 hours. The yeast inocula were diluted to uniform turbidity and 0.1 ml was added to each tube of medium. Actidione was diluted in an agar medium of the following composition: glucose 1.0 per cent, Difco peptone 0.5 per cent, Difco yeast extract 0.1 per cent, and agar 2.0 per cent; pH 7.0. Agar plates of this medium were streaked with spore suspensions of the fungal pathogens, prepared from 2-week-old agar slant cultures, and incubated 72 hours at 30 C. The antibiotic potencies of actidione were recorded as the concentration in micrograms per ml giving complete inhibition of the test organism.

None of the bacteria tested were inhibited by a concentration of 1 mg of actidione per ml. The 12 species tested were as follows: *Aerobacter aerogenes* ATCC 8308, *Bacillus mycoides* ATCC 6462, *Bacillus subtilis* ATCC 6633, *Bacillus subtilis* ATCC 6051, *Escherichia coli* lab. strain, *Phytomonas campestris* ATCC 7381, *Proteus vulgaris* ATCC 220, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella schottmuelleri* ATCC 9149, *Staphylococcus aureus* FDA 209, *Streptococcus faecalis* ATCC 6057, and *Streptococcus pyogenes* C-203. Two of the bacteria, *B. mycoides* and *B. subtilis* ATCC 6633, were obtained from Dr. S. A. Waksman and were the identical strains that are inhibited by Waksman's ether-soluble antibiotic from *S. griseus* (Waksman *et al.*, 1946). The different yeasts varied widely in their response to actidione (table 5). Nine species were completely inhibited by 0.17 microgram per ml; five species were not inhibited by 1,000 micrograms per ml. Of the fungal pathogens only the yeast, *Cryptococcus neoformans*, was very sensitive to actidione (table 6). In this connection, it should be noted that other strains of the same species of pathogens or the parasitic forms of these organisms might exhibit greater sensitivity to actidione than these results would indicate.

TOXICITY OF ACTIDIONE

The LD_{50} for actidione was determined in five different animals by Dr. M. J. VanderBrook of the Pharmacology Department of The Upjohn Company. The acute toxicity was as follows: (1) LD_{50} in rats by subcutaneous injection was 2.7 mg per kg; by intravenous injection, 2.5 mg per kg; (2) LD_{50} in cats by intraperitoneal injection was approximately 4 mg per kg; (3) LD_{50} in rabbits by intravenous injection was 17 mg per kg; (4) LD_{50} in guinea pigs by subcutaneous injection was 60 mg per kg; and (5) LD_{50} in mice by intravenous injection was 150 mg per kg.

TABLE 5
Yeast spectrum of actidione

TEST ORGANISM	μG/ML INHIBITING
Nematospora phaseoli ATCC 2201	0.17
Pichia membranaefaciens ATCC 2254	0.17
Saccharomyces carlsbergensis ATCC 9080	0.17
Saccharomyces ellipsoideus var. burgundy ATCC 4123	0.17
Saccharomyces fragilis ATCC 2360	0.17
Saccharomyces pastorianus ATCC 2366	0.17
Schwanniomyces occidentalis ATCC 2320	0.17
Sporobolomyces salmonicolor ATCC 623	0.17
Torulaspora fermentati ATCC 2551	0.17
Rhodotorula glutinis ATCC 2527	0.31
Hansenia apiculata ATCC 2101	0.62
Hansenula anomala ATCC 8202	2.5
Saccharomyces cerevisiae ATCC 7754	10.0
Torula utilis ATCC 8206	10.0
Asporomyces urae ATCC 7696	25.0
Debaryomyces globosum ATCC 2053	25.0
Schizosaccharomyces pombe ATCC 2476	25.0
Endomyces magnusii ATCC 2105	>1,000
Kloeckera apiculata ATCC 2301	>1,000
Mycotorula roseo-corrallina ATCC 6495	>1,000
Pityrosporum ovale ATCC 7253	>1,000
Saccharomyces lactis ATCC 8635	>1,000

TABLE 6
Fungal pathogen spectrum of actidione

TEST ORGANISM*	μG/ML INHIBITING
Cryptococcus neoformans	0.24
Phialophora verrucosa	12.5
Monosporium apiospermum	25.0
Blastomyces dermatitidis 930	1,000
Candida albicans	>1,000
Coccidioides immitis 819	>1,000
Geotrichum sp.	>1,000
Hormodendrum compactum	>1,000
Nocardia asteroides 653	>1,000
Sporotrichum schenkii	>1,000
Trichophyton rubrum	>1,000
Trichophyton mentagrophytes NIH 640	>1,000
Trichophyton mentagrophytes P	>1,000
Trichophyton mentagrophytes F	>1,000

* The cultures of fungal pathogens with the exception of *T. mentagrophytes* were obtained from Dr. N. F. Conant of Duke University. *T. mentagrophytes* NIH 640 was obtained from Dr. C. W. Emmons of the National Institute of Health.

SUMMARY

Yields of actidione and streptomycin may vary independently of each other with changes in the composition of the culture medium. A mutation that de-

stroys the ability of a strain of *Streptomyces griseus* to synthesize actidione may not have a similar effect upon the yield of streptomycin although the more frequent condition is one in which yields of both actidione and streptomycin are decreased by mutation.

A paper-disk plate method of assay of actidione is described. This assay is characterized by a marked inoculum size effect.

Antibiotic spectra are given for bacteria, yeasts, and fungal pathogens of man. Actidione was inactive against the bacteria tested and highly active against certain yeasts, including the fungal pathogen *Cryptococcus neoformans*.

There is a marked species difference in respect to the toxicity of actidione in animals. The LD_{50} varies from 2.5 mg per kg for rats to 150 mg per kg for mice.

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THE RELATIONSHIP OF VARICELLA AND HERPES ZOSTER: ELECTRON MICROSCOPE STUDIES

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The possible relationship or even identity of the viruses of varicella and herpes zoster was first suggested 40 years ago by von Bokay (1909). Since that time papers too numerous for detailed consideration have appeared in support of, or opposition to, the hypothesis of close relationship or identity. Reviews are to be found in papers by Rivers and Eldridge (1929a,b) and by Amies (1934). In general, it may be stated that today most observers believe that many, if not all, cases of herpes zoster are caused by the virus of varicella, and that they in turn can give rise to new cases of varicella. Kundratitz (1925), by the inoculation of clear fluid from vesicles of herpes zoster, obtained typical zoster lesions, localized clear vesicles on a reddish base, in 14 of 28 children who had not had varicella previously. He failed to transmit the disease to 10 who gave previous histories of varicella. Of the 14 children with no history of varicella who did not develop zoster, 3 showed typical varicella. All inoculations were made into light scarifications on the skin. The incubation period was from 9 to 14 days. Successful inoculation of zoster rendered children immune to subsequent inoculation with vesicular fluid from varicella. Zoster convalescent serum protected exposed children against varicella.

Elementary bodies of varicella were first described in smears of fluid withdrawn from the vesicular lesions by Paschen (1919), and they were subsequently confirmed by Amies (1933) and van Rooyen and Illingworth (1944). Amies noted that the elementary bodies could be agglutinated by convalescent serum from cases of varicella. Ruska (1943) first described the appearance of the elementary bodies of varicella under the electron microscope. He considered them to be round and with a central dot, and separated them from the brickshaped elementary bodies of vaccinia, molluscum contagiosum, and ectromelia. Only drawings were shown, no actual micrographs. Independently of Ruska's work and unaware of it, Nagler and Rake (1948) described the appearance of the bodies of varicella under the electron microscope as brick-shaped and in every way, except that of size, resembling the elementary bodies of the other pox viruses. The average measurements were 210 by 243 mμ, i.e., 15 per cent smaller than those of the elementary bodies of variola or vaccinia. They were infrequent in the vesicular fluids examined.

Paschen (1933) found numerous elementary bodies in vesicular fluids from two cases of herpes zoster. These elementary bodies resembled those found in varicella fluids and could be agglutinated with convalescent serum from cases of both varicella and herpes zoster. Amies (1933) confirmed this and pointed out further that these elementary bodies were plentiful in vesicle fluids 24 to 48 hours after onset (earlier specimens were apparently not examined). Later, as inflammatory cells appeared, the virus particles decreased in number and by the fourth day many of them were clumped together. Amies found a certain degree of cross agglutination between varicella or zoster antisera and elementary body suspensions from the two diseases. We know of no record of electron microscope studies of the elementary bodies of herpes zoster.

MATERIALS AND METHODS

Most of the material used in the present studies came from hospitals of the University of Pennsylvania and was collected by three of the authors (H. B., L. L. C., and T. McN. S.), but some was collected at Bellevue Hospital, New York,[3] and in the Squibb Institute (by F. P. O. N. and G. R.). It consisted of fluid removed from the vesicular lesions of herpes zoster or varicella by means of the capillary attraction of a fine capillary tube. The capillary tubes were sealed for transportation. These fluids were treated in the following manner. The amounts of fluid varied from 0.1 to 0.5 ml. In each case the fluid was diluted to 0.5 ml with distilled water, or, in later studies, with osmic acid of sufficient strength to give a final 1 per cent concentration, and then centrifuged for 20 minutes at 2,000 rpm. The sediment was discarded and the supernatant centrifuged at 15,000 rpm for 1 hour. Both supernatant and sediment were saved for examination, but in general only the sediment gave significant results. It was resuspended in distilled water to the original volume. The suspension of sediment so obtained was placed on collodion membranes and dried in the usual way. In the case of one fluid (S.S.) the technique was varied slightly. A 0.15-ml amount of vesicle fluid was diluted to 0.75 ml with physiological saline. It was then treated as described above except that the final sediment on the screens was washed once with distilled water. Specimens were examined in all cases under an EMU electron microscope by both direct visualization and visualization after use of the shadow-casting technique using gold (Williams and Wyckoff, 1945).

RESULTS

The results of electron microscope studies on vesicle fluids from varicella have been described elsewhere (Nagler and Rake, 1948). Studies carried out with material in the present series have given us even better brick-shaped examples of the varicella elementary bodies than those published hitherto. Typical examples appear in figure 1 and 2. The average size of all the shadowed elementary bodies of varicella we have studied so far is 210 by 243 mμ. The ratio of length

to breadth, 1.16, corresponds to a ratio of 1.24 obtained for both variola and vaccinia, showing that the shape is in general the same for all three elementary bodies. Of course, rounded forms are found in all three cases. Surface irregularities occur as have been described for the other pox viruses, and these undoubtedly might appear as internal dots such as have been described by Ruska (1943).

In our earlier studies with vesicle fluid from herpes zoster very few bodies were seen that could be recognized as of any possible significance. Thus in 7 of 8 fluids collected 24 to 144 hours after the first appearance of the vesicle only single bodies, or on three occasions pairs, were seen after search of several screens. In one case no characteristic bodies were found. The bodies so seen were also brick-shaped (figures 3 and 4) or had a central depression (figure 5). Not enough particles were seen to obtain significant average measurements, but in general the size seemed to be close to that of varicella. At this time we were fortunate enough to learn of a case (S. S.) of herpes zoster in Bellevue Hospital, New York (see footnote 3).

The patient S. S., an adult female, was admitted to Ward 5A, Bellevue Hospital, New York, from the Mental Ward of Bellevue Hospital, suffering from cardiac decompensation. On December 14, 1947, while on a salt-free diet as part of the treatment for her cardiac condition, she complained of severe pain over the left pectoral muscles. A few vesicles appeared in this site on the same day. There had been no complaint of pain prior to the day of the first appearance of rash, but the mental condition of the patient was clouded. Within the next 72 hours new vesicles appeared covering the skin over the left pectoral muscle, the medial part of the left deltoid, and the lower left anterior cervical region. The vesicles were larger than usual, some measuring 1.5 by 1 cm. The first specimen of fluid was withdrawn on December 17, 1947, from unbroken vesicles that had appeared only 12 hours before. It appeared clear. A second specimen was withdrawn 48 hours later from unbroken vesicles of the same crop.

In the 60-hour specimen very few more elementary bodies were seen than in the 7 specimens mentioned above. In the 12-hour preparation, however, quite a different picture was found. Apparent elementary bodies were plentiful. They were found singly (figure 6), in pairs (figure 7), in chains (figure 8), or in clumps (figure 6), and some (figure 9) appeared to be surrounded by a matrix with "sticky" characteristics similar to those described for the Bollinger body of fowl pox (Groupé and Rake, 1947). Bodies with a central depression were also not infrequent (figure 6). In only two respects did these elementary bodies differ from bodies described for varicella; they were usually, but not invariably, equal in any two diameters, i.e., were round, or square with rounded corners, and they were for the most part flatter than usual. It seems possible that these differences were related to the cardiac decompensation and tissue edema since it was found that in a suspension that had stood for 12 days in physiological saline, the virus particles lost their flattened appearance (figures 10, 11, and 12). Measurements of a significantly large number of particles both from S. S. and from other cases gave average measurements of 196 by 218 mμ (range 158 to 251
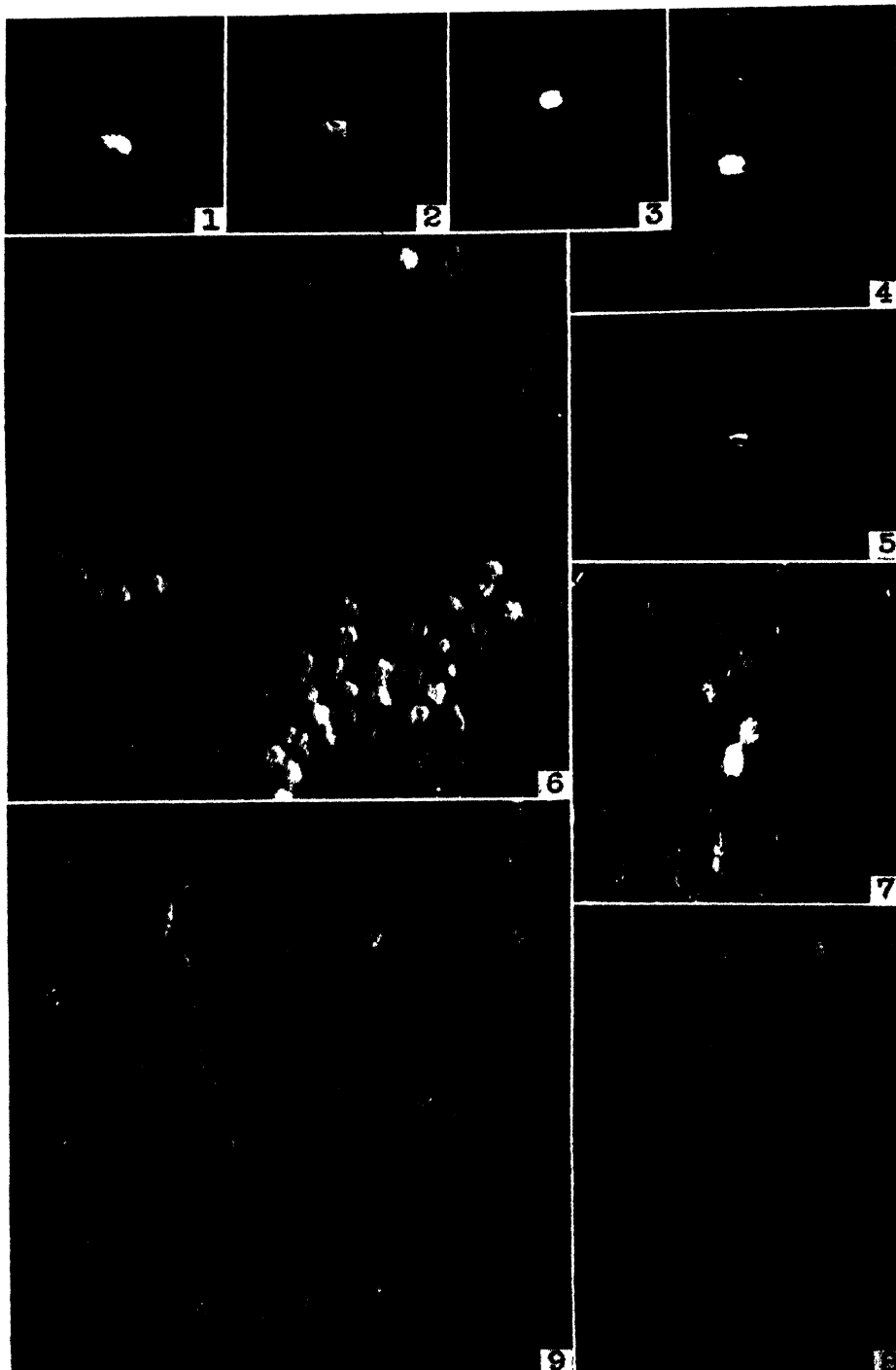
by 186 to 288 mμ) with a ratio of length to breadth of 1.12. These measurements, when compared with those of varicella, are found to be about 8 per cent smaller. In the few particles other than those from S. S. the average diameters were 208 by 240, i.e., identical with varicella, and it may therefore be that, since most particles measured came from case S. S., certain factors in this case, producing shrinking of the bodies, were sufficient to give an over-all figure slightly smaller than that found in varicella. An alternative hypothesis is that the virus bodies gradually acquire a coating of material from the vesicle fluid. If this coating was thin in the case of the 12-hour specimen, the particles would appear to be smaller.

In the original material from S. S. small clumps of elementary bodies were seen (figure 6). The effect of various sera on the suspension of elementary bodies from S. S. was investigated. Serum derived from S. S. (herpes zoster) when she was convalescent, acute and convalescent phase serum from varicella in a child (W. T.) without previous history of herpes zoster, and adult serum from R. D. with a past history of varicella were used. The sera were untreated and all were diluted 1:20 with physiological saline and mixed with the elementary body suspension (0.09 ml of serum + 0.01 ml of virus suspension). This mixture was allowed to stand for 45 minutes at room temperature (22 C) and then centrifuged at 15,000 rpm for 60 minutes at 4 C. The supernatant was discarded and the sediment was resuspended in 0.05 ml of distilled water. These suspensions were used for preparing the screens for examination with the electron microscope. When dried on the collodion membranes, they were washed once with distilled water.

When convalescent varicella or zoster serum was used, very large clumps of elementary bodies were found embedded in a peculiar matrix (? serum) showing many lacunae (figures 13, 14, and 15). However, it was found that adult, and even acute phase serum from a case of varicella, gave some clumping, although the clumps were less frequent and not so large as in the case of convalescent serum

Figure 1. Elementary body of varicella. Note brick shape and irregularities of surface. Shadowed with gold 21.2 mg, angle 11°31′, distance 10 cm   × 17,240.
Figure 2. Elementary body of varicella. Note brick shape and irregularities of surface. Shadowed with gold 21.2 mg, angle 11°31′, distance 10 cm   × 17,240.
Figure 3. Elementary body of herpes zoster. Shadowed with gold 10 4 mg, angle 10°59′, distance 10.5 cm.   × 17,240
Figure 4. Elementary body of herpes zoster. Shadowed with gold 22 4 mg, angle 11°32′, distance 10 cm.   × 17,240.
Figure 5. Elementary body of herpes zoster. From patient J. P.   Shadowed with gold 21.6 mg, angle 11°32′, distance 10 cm.   × 17,240.
Figure 6. Elementary bodies of herpes zoster. One single body, at the top of the figure, and two clumps appear. Several show irregularities of surface and 3 show marked central depressions as in figure 5. Shadowed with gold 27.6 mg, angle 9°36′, distance 12 cm. × 17,240.
Figure 7. Elementary bodies of herpes zoster. One pair attached by corner. Shadowed with gold 26.7 mg, angle 9°36′, distance 12 cm.   × 17,240.
Figure 8. Elementary bodies of herpes zoster. Chain and scattered particles. Shadowed with gold 26.7 mg, angle 9°36′, distance 12 cm.   × 17,240.
Figure 9. Elementary bodies of herpes zoster. Particularly in the upper part of the figure can be seen what appears to be matrix material, with "sticky" characteristics, in which the elementary bodies are embedded. Shadowed with gold 26.7 mg, angle 9°36′, distance 12 cm.   × 17,240.
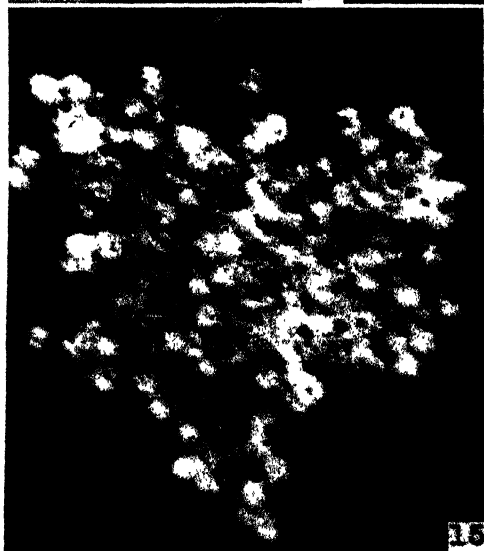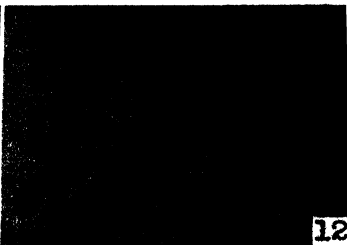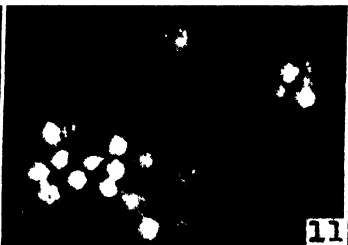
(from varicella or from S. S. herself).   Adult serum, being derived from an individual with a history of varicella many years before, might be expected to give about the same response as that noted, but this does not explain the results with the acute phase serum.   Figure 16 shows the largest clump encountered with adult serum.   Almost as large a clump was seen once in an acute phase serum from a case of varicella (W. T.).   As inspection of these micrographs will reveal, there seems to be a coating of the virus particles in the case of the convalescent serum, giving them a fuzzy appearance, which is much less in the case of the adult serum or the acute phase serum.

It was of great interest that one of the cases of herpes zoster from which vesicle fluid was collected (J. P., see figure 5) was the apparent initiation point of an epidemic of chicken pox, from three of which cases vesicle fluid was collected and examined.[1]

J. P., a colored female aged 6 years, was admitted to the University of Pennsylvania Hospital on August 18, 1947, with chronic infection of the lungs and a chronic nasal discharge.   She had had varicella 1 year before.   On October 10 a group of vesicles appeared on the left side of her back.   They were single or confluent, on an erythematous base, and contained clear fluid.   Their appearance was accompanied by local pain and malaise.   They continued to spread, and after 4 days there was a group of them in a space 3 by 4 inches on the left side of the spine at the level of the third and fourth thoracic vertebrae and a group extending into the left axilla and to the midline above the left nipple.   On October 14, when fluid was collected for electron microscopy, a biopsy of a skin vesicle was taken, fixed in Bouin's solution, and stained with hematoxylin and eosin.   Type A inclusion bodies were seen in the epithelial cells lining the vesicle (figure 17).

At the time J. P. first developed herpes zoster there were 18 other children in the ward, and during her zoster illness a total of 27 were exposed.   Of these, 22

Figure 10 Elementary bodies of herpes zoster after standing for 12 days in physiological saline.   Note plumper appearance as compared, for example, with those in figure 6 Shadowed with gold 9 6 mg, angle 10°59', distance 10.5 cm.   × 17,240

Figure 11. Elementary bodies of herpes zoster   As in figure 10.   Shadowed with gold 9 6 mg, angle 10°59', distance 10.5 cm.   × 17,240.

Figure 12. Elementary bodies of herpes zoster after standing for 12 days in physiological saline but without added serum.   Unshadowed   × 17,240.

Figure 13. Agglutinated elementary bodies of herpes zoster.   With convalescent serum. Note indistinct outline of all virus particles, even those somewhat removed from agglutinated mass.   Note also lacunae in the serum-virus complex probably due to washing out of crystals.   Unshadowed.   × 17,240.

Figure 14. Agglutinated elementary bodies of herpes zoster.   With convalescent serum. As in figure 13.   Shadowed with gold 9.0 mg, angle 10°59', distance 10.5 cm   × 17,240.

Figure 15. Agglutinated elementary bodies of herpes zoster.   With convalescent serum. As in figure 13.   Shadowed with gold 9.0 mg, angle 10°59', distance 10.5 cm.   × 17,240

Figure 16. Clumped elementary bodies of herpes zoster.   With adult serum.   This was the largest clump found.   Compare with figures 14 and 15.   Note clear outline of virus particles and less evidence of serum in and around the clump.   Shadowed with gold 9.0 mg, angle 10°59', distance 10.5 cm.   × 17,240.

had never had varicella, and of the 22, 10 developed typical varicella from 15 to 19 days after the first day of rash in J. P.   Three of these 10 developed in the

Figure 17. Biopsy of herpes zoster lesion from J. P.   Type A intranuclear inclusions are seen in the epithelial cells lining the vesicle.   Haematoxylin and eosin stain.   × 900.

hospital ward and 10 additional susceptible children were exposed.   Of these 10, 3 developed varicella 15 to 20 days later.   Fluids from three cases were examined

with the electron microscope. Typical if scanty brick-shaped elementary bodies were seen in all three fluids.

Two other patients are worthy of comment as representing examples of "traumatic herpes zoster." From both of these, vesicle fluid was studied and found to contain the characteristic bodies. The first case is one of traumatic herpes following lumbar puncture.

C. B., a 3½-year-old colored boy, was admitted to the Philadelphia Hospital for Contagious Diseases on August 20, 1947, with a diagnosis of miliary tuberculosis. Subsequently, he developed signs of meningeal irritation, and intrathecal streptomycin was instituted on December 6, 1947, and continued daily until January 16, 1948, when a regime of intrathecal treatment on alternate days was adopted. On January 22, 1948, he developed a band of grouped vesicular lesions on an erythematous base extending in segmental nerve distribution on the left side from the third and fourth lumbar vertebrae to the midline over the abdomen. He had never had varicella. There had been no cases of varicella or herpes zoster in the building for a period of 7 months. None of the contacts of C. B. contracted either varicella or herpes zoster. Spinal fluid collected on January 22, 1948, and vesicle fluid collected on January 24, 1948, were examined under the electron microscope.

The second case is one of traumatic herpes following deep irradiation for carcinoma of the cervix. M. T., a 59-year-old white woman, from November 24 to December 23, 1947, received three times weekly 1,500 R of deep X-ray therapy directed over pubic and sacral areas for carcinoma of cervix. On December 15, 1947, vesicles appeared on the pubis and sacrum. The clinical impression was herpes simplex or possibly herpes zoster. Vesicle fluid was collected on December 15, 1947, for electron microscope study. Scanty bodies characteristic of herpes zoster were found in both vesicle fluids. Figure 3 shows a single body from C. B. The spinal fluid of C. B. showed no such bodies.

<div align="center">DISCUSSION</div>

In general, then, elementary bodies have been found in vesicle fluid from the lesions of herpes zoster that differ little if at all from those described for varicella either in size or in shape. It is suggested that the slightly smaller size (8 per cent) of the zoster bodies may have been due to abnormal tonicity relations in the surrounding fluid in the case of S. S., from whom most of the particles were derived.

The observations in the case of S. S. are of particular importance. It will be noted that enormous numbers of elementary bodies were present in the vesicles 12 hours after the appearance of these vesicles, but 72 hours after the first onset of the disease. At this time a certain amount of clumping (Amies, 1934) was already present. Forty-eight hours later most of the virus particles had disappeared, and those seen were for the greater part embedded in ill-defined masses of material of low density suggesting some protein material derived from body fluids. The present observations do not resolve the difficult question of the pathogenesis of herpes zoster. Certain points, however, are worth commenting

on in this regard. One is the finding of large numbers of virus particles in the early vesicle fluid and only a few in the later vesicle fluid (S. S.). This would suggest that virus multiplied in the skin early in the development of the lesions. The fact of virus multiplication in the skin is certainly also suggested by the histological picture, since the characteristic inclusion bodies considered indicative of the presence of virus are always found in this organ (figure 17). Finally, there is the finding of the same type of elementary bodies in the vesicles of all the patients studied whether they were suffering from the so-called primary herpes zoster, either sporadic or associated with a case of varicella (for example, S. S. and J. P.), or from herpes zoster secondary to trauma (for example, C. B. and M. T.). It is interesting to note that the site of eruption of the traumatic cases conformed to the area of skin damaged. C. B. had lesions at the level of the third to fourth lumbar vertebrae, i.e., at the level of the lumbar punctures, and not along the distribution of the segmental nerves from that region. It is conceivable that the meningitic process could have affected the nerve roots supplying the involved skin area, but this possibility is rendered unlikely by the observation that traumatic zoster, following lumbar puncture for reasons other than meningitis, regularly occurs at the site of the puncture. M. T. showed the same phenomenon, the site of X-ray application and not the site of nerve distribution from the sacral area being affected. It would seem reasonable to assume that the virus causing herpes zoster probably multiplies in the skin. Whether it multiplies elsewhere, and what the relationship is to the central nervous system changes frequently described, still demand further investigation. It would also appear probable that only one virus is responsible for the clinical types of herpes zoster we have studied and that this virus is identical with that of varicella.

Amies (1934) noted that clumping of the virus particles occurred in zoster vesicles 96 hours after first appearance. In the present case some clumping was apparent even in the 12-hour fluid. It is possible that this may be due to small amounts of circulating antibody from a previous attack of varicella and to an accelerated serological response during the 72 hours the disease had been in progress. Marked agglutination occurred with convalescent zoster or varicella sera, and there was evidence of some coating of the virus particles. However, in the case of the suspension of particles from S. S., the only one we have been able to examine, some agglutination also occurred not only with adult serum but also with acute phase serum. The lacunae noted as constantly present in the serum-elementary-body complexes apparently represent the spaces left by crystals of salt that have been washed out.

The epidemic of varicella apparently initiated by the case of herpes zoster in J. P. can be duplicated by many reports in the literature. The only additional fact of interest in the present account is that typical and similar elementary bodies were found in the vesicles both of J. P. and of the secondary cases of varicella. Because of this, and the temporal relationship of the onset of the first wave of 10 cases to the appearance of the disease in J. P., one can hardly doubt that in this instance both diseases were produced by the same virus.

SUMMARY

Elementary bodies have been demonstrated in the vesicle fluid of the lesions of various clinical types of herpes zoster that resemble closely those found in varicella. In the limited experience so far obtained these elementary bodies have been very scanty in fluids withdrawn from 24 hours to 6 days after the first appearance of the rash. In one case in which fluid was obtained 12 hours after the appearance of the vesicles the elementary bodies were very numerous. Fluid from this same crop of vesicles, withdrawn 48 hours later, showed very few elementary bodies. The possible significance of this is discussed.

In one instance vesicle fluid was examined from a case of herpes zoster that apparently initiated an outbreak of varicella. Fluid was also examined from 3 of 13 cases of varicella. Scanty but characteristic brick-shaped bodies were found in all four fluids.

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OBSERVATIONS ON STREPTOMYCES GRISEUS

II. Nitrogen Sources for Growth and Streptomycin Production[1]

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Synthetic mediums are extremely useful in the investigation of fermentation problems. Because of this fact, various laboratories that are interested in streptomycin have been engaged in developing synthetic mediums for the production of this antibiotic.

VanderBrook *et al.* (1946) reported the use of a near synthetic medium composed of glucose, ammonium sulfate, mineral salts, and 0.1 per cent "curbay B-G." Saunders and Sylvester (1947) used a synthetic medium containing glucose, an organic acid, an inorganic nitrogen compound, and mineral salts. The synthetic medium of Hubbard and Thornberry (1946) was composed of a carbohydrate, the ammonium salt of lactic acid, and mineral salts. It will be noted that these mediums contain inorganic nitrogen compounds or the ammonium salt of an organic acid as the nitrogen source. Although these mediums supported growth and streptomycin production by *Streptomyces griseus*, the reported broth potencies thus obtained were not exceptionally high.

This paper deals with various compounds, both organic and inorganic, as possible nitrogen sources for growth and streptomycin production in a synthetic medium.

MATERIALS AND METHODS

The culture of *Streptomyces griseus* used in these experiments was isolated from a strain originally obtained from Waksman's laboratory. Stock cultures were prepared by adding spore suspensions to tubes of sterile soil. Cultures for inocula were obtained by spreading spores from a soil tube over Blake bottle slants of yeast extract glucose agar. All cultures were derived from the same soil tube. After 7 days' incubation at 28 C, 50 ml of sterile, distilled water were added to each Blake bottle culture and a spore suspension was prepared. One ml of this suspension served as an inoculum for each flask.

The fermentation medium contained the following constituents: glucose 10.0 g, nitrogen source as indicated, NaCl 5.0 g, K_2HPO_4 2.0 g, $MgSO_4 \cdot 7 H_2O$ 1.0 g, $CaCl_2$ 0.4 g, $FeSO_4 \cdot 7 H_2O$ 20 mg, $ZnSO_4 \cdot 7 H_2O$ 10 mg, and distilled water 1 liter. This medium was dispensed in 40-ml amounts in 125-ml Erlenmeyer flasks and sterilised by· autoclaving at 121 C for 17 minutes. The pH was adjusted to between 7.0 and 7.5 with N NaOH or N HCl before autoclaving and rechecked after autoclaving. No medium with a pH below 6.5 was used.

After inoculation, the fermentation flasks were incubated at 28 C on a rotary type shaker moving at 220 rpm so that it described a circle 1 inch in diameter. After growth had started, the fermentation broths were sampled and assayed daily until the maximum streptomycin broth potency had been passed. The agar plate method using a streptomycin calcium chloride complex standard was employed for assay throughout the experiments.

EXPERIMENTAL DATA

Inorganic nitrogen compounds. In order to obtain the simplest medium, the inorganic nitrogen compounds were investigated first. Five compounds, i.e., $NaNO_2$, NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$, were used as single nitrogen sources in the medium noted above at a level of 0.5 per cent. In addition, $CaCO_3$ was added at a level of 0.35 per cent to other replicates except those

TABLE 1

Inorganic nitrogen sources

NITROGEN SOURCE	STREPTOMYCIN BROTH POTENCY—μG PER ML AFTER					
	3 days	4 days	5 days	6 days	7 days	8 days
$NaNO_3$	No growth					
$NaNO_3$ + $CaCO_3$	No growth					
NH_4NO_3	3	6	4	4	4	3
NH_4NO_3 + $CaCO_3$	13	16	20	45	59	64
NH_4Cl	0	0	0	0	0	0
NH_4Cl + $CaCO_3$	8	14	19	32	22	—
$(NH_4)_2SO_4$	6	4	3	3	2	2
$(NH_4)_2SO_4$ + $CaCO_3$	7	7	8	23	29	29
$(NH_4)_2HPO_4$	—	142	178	155	155	—

containing $(NH_4)_2HPO_4$. The streptomycin broth potencies obtained in these fermentation mediums are given in table 1; the specific pH changes are omitted.

Streptomyces griseus will not utilize nitrate as a sole source of nitrogen, hence no growth occurred in the medium containing $NaNO_3$ as the nitrogen source. Ammonia, however, is readily utilized. Thus, in the medium containing NH_4-NO_3 as the sole nitrogen source, the ammonium nitrogen is utilized with a resultant release of the nitrate. The organism grows until the medium becomes too acid for further growth. Comparable growth and pH changes occur in the mediums containing NH_4Cl and $(NH_4)_2SO_4$ as individual nitrogen sources. The addition of $CaCO_3$ to the fermentations keeps the pH relatively high and allows the organism to grow and produce streptomycin, though at relatively low levels. When $(NH_4)_2HPO_4$ serves as the sole nitrogen source, the pH remains relatively high and yields of approximately 150 μg per ml are consistently obtained. The level of phosphate in this medium is quite important. The use of $(NH_4)_2HPO_4$ at a level of 1.0 per cent, with the fermentation conditions used, will result in little or no streptomycin formation. This phosphate effect is interesting and warrants further investigation. It apparently is not a pH effect. In practice the $(NH_4)_2HPO_4$ is employed at a level of 4.0 g per liter.

Effect of adding complex organic materials to the glucose $(NH_4)_2HPO_4$ *medium.*
The addition of various complex organic materials at a level of 0.1 per cent to the
glucose $(NH_4)_2HPO_4$ medium has a marked effect on the fermentation.   Growth
occurs more readily, there is an earlier increase in streptomycin broth potency,
and significantly higher streptomycin levels are reached.   The effect of these
organic materials on the yields of streptomycin can be seen in table 2.   The most
remarkable increase in broth potency has been obtained by the addition of corn
steep solids.   The addition of only 0.1 per cent of such solids results in an
increase in the streptomycin broth potency of approximately 100 per cent.

The stimulation obtained by the addition of corn steep solids to the glucose
$(NH_4)_2HPO_4$ medium cannot be reproduced by the substitution of an equivalent
amount of corn steep ash for the corn steep solids.   The addition of 0.1 per cent
yeast nucleic acid to the glucose $(NH_4)_2HPO_4$ medium also has no stimulating
effect.   Moreover, the strain of *Streptomyces griseus* used in these experiments
exhibits no vitamin deficiency.   Small amounts of a number of organic acids,
namely, acetic, lactic, fumaric, succinic, pyruvic, malic, and citric also were

TABLE 2

Effect of adding complex organic materials to the glucose $(NH_4)_2HPO_4$ medium

MATERIAL ADDED, 0.1 PER CENT	STREPTOMYCIN BROTH POTENCY—μG PER ML AFTER					
	3 days	4 days	4 days	6 days	7 days	8 days
Soybean meal	83	139	200	210	230	215
Corn steep solids	119	228	303	210	—	—
Trypsin digested casein.	112	217	224	249	252	—
None	24	135	153	151	—	—

added individually to the medium.   In no instance was a stimulation of strepto-
mycin production observed.

The possibility exists that this stimulation is due to organic nitrogen com-
pounds in the corn steep, perhaps a single amino acid or a combination of amino
acids.   This possibility was investigated by adding 21 amino acids, creatine,
urea, and guanidine separately at a level of 0.1 per cent to the glucose
$(NH_4)_2HPO_4$ medium.   The results are given in table 3.

It may be noted that the medium to which no organic nitrogen compound was
added supported streptomycin yields of 153 μg per ml.   The marked stimulation
due to the addition of corn steep solids and trypsin-digested casein was again
obtained.   None of the other compounds had a stimulatory effect.   Growth was
sparse in the flasks supplemented with cysteine and DL-norleucine.

These data indicate that the stimulatory effect of the corn steep solids is not
due to a single amino acid that they contain.   However, the stimulation may be
due to a specific combination of amino acids.

Organic nitrogen compounds.   Although individual amino acids had no stimu-
latory effect when added at low concentrations to the glucose $(NH_4)_2HPO_4$
medium, this does not exclude the possibility that some of these compounds could
replace the $(NH_4)_2HPO_4$ in this medium.   The utilization of 33 organic nitrogen

compounds as possible nitrogen sources in this synthetic medium was thus investigated by substituting them individually for the $(NH_4)_2HPO_4$. These compounds were used at a level of 0.186 per cent nitrogen. The $(NH_4)_2HPO_4$ was used at a level of 0.085 per cent nitrogen. The streptomycin yields obtained in these fermentations are given in table 4.[2]

TABLE 3

Effect of adding single organic nitrogen compounds to the glucose $(NH_4)_2HPO_4$ medium

COMPOUND ADDED, 0.1 PER CENT	MAXIMUM STREPTOMYCIN BROTH POTENCY	DAY OF MAXIMUM
	μg per ml	
DL-Alpha-alanine	155	5
Glycine	116	7
L-Arginine HCl	135	6
L-Aspartic acid	125	6
L-Cysteine HCl	0	—
L-Cystine	105	5
Creatine hydrate	163	7
L-Glutamic acid	115	5
L-Histidine HCl	145	5
Hydroxy-L-proline	135	6
DL-Isoleucine	127	7
L-Leucine	137	5
DL-Lysine HCl	139	6
DL-Methionine	84	5
DL-Norleucine	0	—
DL-Phenylalanine	166	7
L-Proline	175	5
DL-Serine	155	5
DL-Threonine	110	7
DL-Tryptophan	129	7
L-Tyrosine	176	6
DL-Valine	168	5
Urea	136	6
Guanidine nitrate	133	6
0.1 per cent corn steep solids	300	5
0.1 per cent casein digest	249	6
None	153	5

A yield of 166 μg per ml was obtained in the medium containing $(NH_4)_2HPO_4$. Of the 33 organic nitrogen compounds, 16 supported growth and streptomycin production. However, only 6 of these compounds, i.e., DL-alpha-alanine, beta-alanine, L-histidine HCl, glycine, L-proline, and L-arginine HCl, supported yields in excess of 100 μg per ml.

The use of glycine as the sole nitrogen source resulted in a yield of 151 μg per

[2] In previous experiments Dr. L. E. McDaniel of the Merck Laboratories had tested proline and a number of other amino acids as single nitrogen sources in a synthetic streptomycin fermentation medium.

ml. However, if phenylglyc*ne or *para*-hydroxyphenylglycine is substituted for glycine, no growth occurs.

In the medium containing alpha-alanine (alpha-amino-propionic acid), streptomycin broth potencies of 234 µg per ml were obtained. Beta-alanine

TABLE 4

Organic nitrogen compounds

NITROGEN SOURCE	MAXIMUM STREPTOMYCIN BROTH POTENCY	DAY OF MAXIMUM
	µg per ml	
DL-Alpha-alanine	234	5
Beta-alanine	221	8
DL-Benzoylalanine	Very slight growth	—
Glycine	151	5
N-Phenylglycine	No growth	—
p-Hydroxyphenylglycine	No growth	—
L-Arginine HCl	101	10
L-Aspartic acid	12	5
DL-Alpha-amino-N-butyric acid	Very slight growth	—
DL-Alpha-amino-caprylic acid	Very slight growth	—
L-Cysteine HCl	0	—
L-Cystine	0	—
Creatine hydrate	0	—
L-Glutamic acid	4	4
Hippuric acid	Very slight growth	—
L-Histidine HCl	112	4
Hydroxy-L-proline	1–2	6
DL-Isoleucine	39	5
L-Leucine	5	6
DL-Lysine HCl	0	—
DL-Methionine	0	—
DL-Norleucine	0	—
DL-Phenylalanine	0	—
L-Proline	800	9
Taurine	Growth very poor	—
Sarcosine	No growth	—
DL-Serine	3	8
DL-Threonine	5	6
DL-Tryptophan	0	—
DL-Tyrosine	0	—
DL-Valine	3	8
Urea	3	6
Guanidine nitrate	5	6
$(NH_4)_2HPO_4$	166	5

(beta-aminopropionic acid) supported streptomycin yields of 221 µg per ml, though maximum titers were reached later than when alpha-alanine was used. Any substitution at the third carbon in alanine resulted in lowered yields. For example, L-histidine HCl (beta-imidazole-alpha-aminopropionic acid) supported

streptomycin yields of only 112 μg per ml.   The addition of the imidazole ring resulted in a decrease in potency of approximately 50 per cent.   DL-Serine (beta-hydroxy-alpha-aminopropionic acid), DL-valine, (beta-dimethyl-alpha-aminopropionic acid), and DL-isoleucine (beta-methyl-beta-ethyl-alpha-aminopropionic acid) supported even lower streptomycin broth titers.

When L-arginine HCl was used as the sole nitrogen source, growth was slow and maximum yields of streptomycin were reached late.   It should be noted that arginine is the only amino acid containing more than three carbons in a straight chain that supports relatively high yields of streptomycin.

Striking results were obtained by the use of L-proline as the sole nitrogen source.   There was an increase in mycelium weight, and streptomycin broth potencies of 800 μg per ml were obtained.   There was, however, a lag in both growth and streptomycin production.

Attempts to substitute structurally related compounds for the proline have met with failure.   The use of hydroxyproline as the sole nitrogen source results in poor growth and little if any streptomycin production.   Pyrrolidone-carboxylic acid and N-acetyl proline, when substituted for the L-proline, do not support growth.   The same is true for pyrrole.

It has been shown that the ornithine cycle proposed by Krebs and Henseleit (1932) exists in *Neurospora crassa* (Srb and Horowitz, 1944).   Moreover, the metabolism of proline in the rat (Shemin and Rittenberg, 1945; Stettin and Schoenheimer, 1944) is closely linked to this ornithine cycle.   In addition, Bonner (1946) indicates that in *Penicillium* ornithine and proline have a common precursor, with glutamic acid the precursor of this proposed intermediate.   He represents the synthesis of proline and arginine in *Penicillium* by the following scheme:

glutamic acid   →   intermediate   →   ornithine   →   citrulline   →   arginine
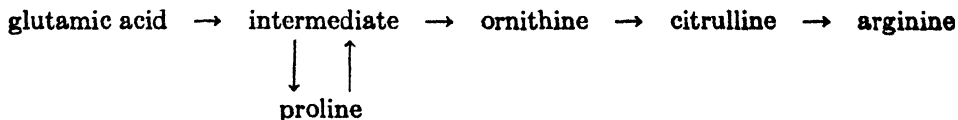
proline

It would appear that this scheme does not hold true for *Streptomyces griseus*. Glutamic acid, when substituted for proline, supports good growth but variable and usually low streptomycin yields.   The use of arginine as the sole nitrogen source results in fair growth and streptomycin broth potencies of approximately 100 μg per ml.   None of the other valeric derivatives has even supported growth in mediums to which it was added as an individual nitrogen source.   Alpha-aminovaleric acid, delta-aminovaleric acid, and alpha-delta-diaminovaleric acid (ornithine) were the compounds tested.   In addition, N-valeric acid when used as the sole carbon source in a medium containing $(NH_4)_2HPO_4$ as the source of nitrogen did not support growth.

The high broth potencies that have been obtained in the proline medium raise the possibility of adding proline to other mediums and thus obtaining increased streptomycin yields in these mediums.   In preliminary experiments, the addition of proline at a level of 0.25 per cent to a medium containing $(NH_4)_2HPO_4$, casein

digest, or soybean meal as the nitrogen source resulted in no increase in streptomycin broth titers. The results reported in a previous section showed that single amino acids, when added at a level of 0.1 per cent to the glucose $(NH_4)_2HPO_4$ medium, did not increase streptomycin broth potencies. In addition, L-proline, glycine, DL-alpha-alanine, L-arginine HCl and L-histidine HCl were added singly to the glucose $(NH_4)_2HPO_4$ medium at a level of 0.5 per cent. All possible combinations of these compounds also were added to the same medium in such quantities that the total amount of added amino acids was always 0.5 per cent. Casein digest and proline-rich gelatin also were tested. The yields of streptomycin obtained in these fermentations are shown in table 5.

TABLE 5

The effect of adding amino acids singly and in combination to the glucose $(NH_4)_2HPO_4$ medium

MATERIAL	TOTAL AMOUNT	MAXIMUM STREPTOMYCIN BROTH POTENCY	DAY OF MAXIMUM
	per cent	*µg per ml*	
DL-Alpha-alanine..	0.5	56	5
L-Arginine HCl.	0.5	61	6
Glycine	0.5	68	6
L-Proline........	0.5	170	6
L-Histidine HCl......	0.5	56	6
Proline, alanine, glycine, histidine, arginine	0.5	51	6
Proline, alanine, glycine, histidine........	0.5	55	5
Proline, alanine, glycine..	0.5	51	6
Proline, alanine	0.5	59	5
Alanine, glycine, histidine, arginine.....	0.5	41	5
Alanine, glycine, histidine..	0.5	51	5
Alanine, glycine	0.5	30	5
Glycine, histidine, arginine	0.5	31	5
Glycine, histidine.....	0.5	66	6
Histidine, arginine.	0.5	50	5
Casein digest	0.5	275	5
Casein digest	0.3	274	5
Gelatin	0.5	195	5
None...	—	144	6

With the exception of L-proline at 0.5 per cent the addition of the amino acids, singly and in combination, resulted in decreased streptomycin yields. The increased potencies due to the addition of 0.5 per cent L-proline is significant but not striking. Gelatin at 0.5 per cent also increased yields significantly as did casein digest at 0.3 per cent and 0.5 per cent. The increase due to the use of casein digest is marked. The addition of compounds that are metabolically related to proline, e.g., ornithine, has not resulted in a stimulation of streptomycin production.

Although no specific data are given, it also should be noted that, when other nitrogenous compounds are added to the synthetic medium containing L-proline as the principal source of nitrogen, the streptomycin yields thus obtained are

much lower than are obtained with proline alone. This is not true if the level of added nitrogenous compounds is very low.

These data certainly indicate that L-proline when used as the sole nitrogen source is capable of supporting high streptomycin broth potencies. Although it is true that increased mycelium weights are obtained in this L-proline medium, excellent growth can be obtained in mediums that support little or no streptomycin formation.

The data presented thus far raise a number of interesting and important questions, the answers to which will have to await the results of metabolism studies now in progress. Of particular importance will be the mechanism of proline utilization in different mediums, i.e., a medium in which proline is the sole nitrogen source and a medium in which it is used in conjunction with other nitrogen compounds, such as $(NH_4)_2HPO_4$.

SUMMARY

A number of organic and inorganic nitrogen compounds have been tested as possible nitrogen sources in a synthetic streptomycin fermentation medium.

Nitrate nitrogen is not utilized by the streptomycin-producing strains of *Streptomyces griseus* that have been studied, but ammonium nitrogen is readily available.

A simple synthetic medium containing ammonium nitrogen has been devised. This medium contains glucose 10.0 g, $(NH_4)_2HPO_4$ 4.0 g, NaCl 5.0 g, K_2HPO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $CaCl_2$ 0.4 g, $FeSO_4 \cdot 7H_2O$ 20.0 mg, $ZnSO_4 \cdot 7H_2O$ 10.0 mg, and distilled water 1 liter. Streptomycin broth potencies of 150 μg per ml are consistently obtained in this medium. The level of phosphate is important.

The addition of small amounts of corn steep solids, casein digest, and soybean meal to this medium results in increased titers of streptomycin. If amino acids are added singly to this medium, there is no stimulation of streptomycin production.

Thirty-three organic nitrogen compounds were tested as possible substitutes for the $(NH_4)_2HPO_4$ in the synthetic medium. Six of these compounds, namely, DL-alpha-alanine, beta-alanine, L-histidine HCl, L-arginine HCl, glycine, and L-proline, supported yields of streptomycin in excess of 100 μg per ml. Yields of 800 μg per ml were obtained by the use of L-proline as the sole nitrogen source.

The use of L-proline as a supplement did not increase streptomycin broth potencies.

A number of compounds that are structurally related to L-proline have been tested as substitutes for the proline. None of them could replace this amino acid.

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STUDIES ON THE PHYSIOLOGY OF A STREPTOMYCIN-PRODUCING STRAIN OF STREPTOMYCES GRISEUS ON PROLINE MEDIUM

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Complex organic mediums have generally been considered necessary for the production of high yields of streptomycin by *Streptomyces griseus* (Waksman, Schatz, and Reilly, 1946). Quantities of streptomycin approaching 1 gram per liter have been reported from the growth of mutant strains on such mediums (Stanley, 1947). This represents a conversion of 5 to 10 per cent of the organic constituents of the medium to streptomycin. Few attempts have been made to define conditions regulating the conversion or to study the mechanism of streptomycin formation.

Dulaney and Perlman (1947) have reported observations of the biochemical activities of *S. griseus* under conditions favorable for streptomycin production. Two stages of activity in a glucose peptone meat extract medium were described. During the growth phase, the production of mycelium was accompanied by a reduction in the soluble constituents of the medium (N, C, P), fermentation of the available carbohydrate, a high oxygen demand, and little production of streptomycin. During the autolytic phase, the mycelium weight decreased markedly, inorganic phosphorus and soluble nitrogen were released into the medium, the oxygen demand dropped, and considerable quantities of streptomycin were produced.

In the preceding paper from this laboratory (Dulaney, 1948), a synthetic medium containing proline is described that supports streptomycin production equal to or better than that obtained with organic mediums. The stages of the biochemical activities of *S. griseus* on this medium are outlined in figure 1. The first stage, that of cell growth not associated with streptomycin production, is prolonged in the proline medium, primarily because of an extended lag phase before proline is attacked or growth occurs. In phase 2 the remaining amounts of glucose and proline are utilized, accompanied by rapid accumulation of streptomycin. Finally, lysis of the culture occurs.

The specific action of proline, among amino acids, in allowing high streptomycin yields, offers the opportunity for study of the mechanism of streptomycin formation. As a part of this investigation, methods were sought to decrease the lag phase of growth. These included an attempt to promote growth by the utilization of adapted *S. griseus* inoculum, the addition of essential nutrients, and the study of products intermediate in proline decomposition that were more susceptible than proline to attack by *S. griseus*, but were still conducive to high streptomycin yields.

Analytical methods. With the exception of the determination of proline, standard analytical procedures were used. Glucose was determined as a re-

ducing sugar by the method of Somogyi, total nitrogen by micro-Kjeldahl procedure, NH_4-N by steam distillation from 5 per cent Na_2CO_3 solution, and total carbon by the wet combustion method of McCready and Hassid (1942). Streptomycin was determined by the FDA cup assay. Proline determinations were made by an unpublished procedure, developed in our laboratories and based on the amino acid assay procedures described by Stokes, Gunness, Dwyer, and Caswell (1945). *Leuconostoc mesenteroides* was employed as the assay organism.

Most of the fermentations were conducted in 250-ml Erlenmeyer flasks containing 25 ml of medium and were incubated at 28 C on a rotary shaker operating
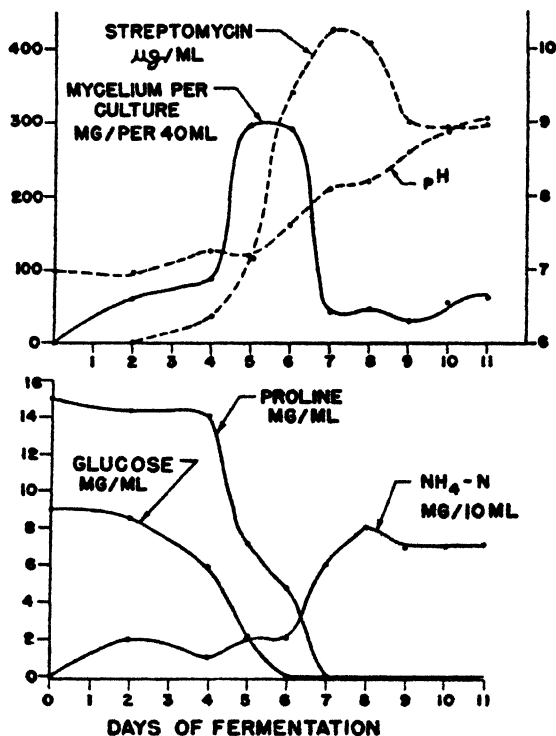
Figure 1. Fermentation characteristics of *S. griseus*.

at 220 rpm. The *S. griseus* inoculum was derived from strains previously found satisfactory for streptomycin production (Schatz and Waksman, 1945).

EXPERIMENTAL PROCEDURE AND RESULTS

Development of "adapted" cells. *S. griseus* was grown from a spore inoculum on a synthetic basal medium containing 0.57 per cent $(NH_4)_2HPO_4$ or on 1 per cent proline, which contains an equivalent amount of nitrogen (Dulaney, 1948). Mycelial growth was considered suitable for transfer after attaining about 80 per cent of its maximum weight.

An attempt to show the adaptability of the organism to proline was made by

10 per cent transfer of vegetative inoculum to fresh culture medium (table 1). There was no evidence that cells became adapted to use proline. Cells developed on proline had a lag phase equal to that of those developed on $(NH_4)_2$HPO$_4$ medium, when inoculated into proline fermentation medium. Equivalent results were obtained when 100 per cent transfer was made of washed cells. The lag was much reduced, but equal in the case of inoculum developed on either $(NH_4)_2$HPO$_4$ or proline medium.

A striking difference was noticed in streptomycin yields from fermentations initiated with the two types of vegetative inocula. Only those cultures receiving the inoculum developed on proline medium produced high streptomycin yield. This suggests that the specific action of proline in increasing streptomycin production is not due to a direct precursor action but results from a more general effect on the physiological activity of the cellular substrate.

TABLE 1

The effect of the medium used for inoculum development on streptomycin production and proline utilization

INOCULUM DEVELOPMENT			FERMENTATION IN PROLINE MEDIUM	
Nitrogen constituent	Maximum streptomycin produced	Inoculum size	Streptomycin produced	Day of proline utilization
	µg/ml	*per cent*	*µg/ml*	
$(NH_4)_2$HPO$_4$*	175	10	200	3
Proline†	960	10	930	3½
$(NH_4)_2$HPO$_4$	115	100	375	2½
Proline....	930	100	525	2½

* Used as vegetative inoculum on fourth day.
† Used as vegetative inoculum on seventh day.

Requirement for NH$_4$-N. The synthetic basal medium, containing a mixed N source composed of $(NH_4)_2$HPO$_4$ and proline, showed no lag in proline utilization, despite the fact that there was ample nitrogen present as NH$_4$-N for growth of the culture. Complete proline decomposition, NH$_4$-N utilization, and maximum growth coincided on the fourth day of incubation from a spore inoculum, as compared with seven days on proline alone. *S. griseus* has great difficulty in utilizing proline for the supply of its total nitrogen demands. However, if a trace quantity of NH$_4$-N is present to supply an essential requirement for unknown synthetic processes, proline can adequately supply the major portion of the nitrogen needs of the microorganism. A correct balance of NH$_4$-N can be found that will speed the rate of growth of *S. griseus* to a maximum but will not interfere with the development of mycelium of high streptomycin-producing capacity. An optimum of 0.02 per cent $(NH_4)_2$HPO$_4$ with 1 per cent proline was found (table 2).

Carbon-nitrogen balance. Proline utilization, once initiated, is very rapid. NH$_4$-N does not accumulate during the decomposition. It is evident that intermediate nitrogenous compounds must accumulate in the medium. During the

utilization of these intermediates streptomycin production occurs. Graphs representing the carbon and nitrogen balance at various incubation periods in the proline fermentation are shown in figures 2 and 3. The medium was the proline medium described in the preceding paper (Dulaney, 1948), which contained 1.5

TABLE 2

Streptomycin production on a proline medium with the amount of $(NH_4)_2HPO_4$ as a variable

(NH$_4$)$_2$HPO$_4$ CONCENTRATION	STREPTOMYCIN YIELD ON 8TH DAY
per cent	*μg/ml*
0.57	225
0.20	170
0.06	570
0.02	675
0.006	530
0.002	390
0.0006	200
0.0002	130
—*	25

(NH$_4$)$_2$HPO$_4$ medium control, maximum on seventh day, 120 μg.

* Maximum on proline medium was reached on eleventh day of incubation, 430 μg.
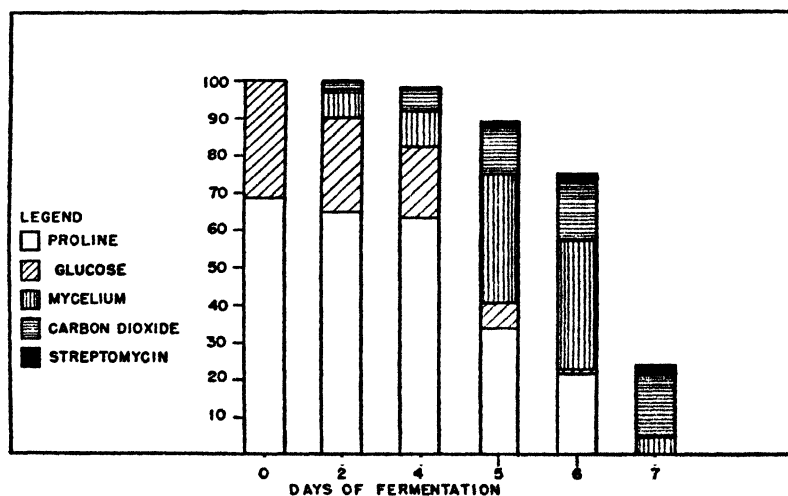
Figure 2. Carbon balance of *S. griseus* fermentation.

per cent L-proline and 1 per cent glucose. During the maximum rate of mycelium accumulation, between the fourth and sixth day, unaccounted-for nitrogen was present. An associated quantity of unaccounted-for carbon also points to the accumulation of intermediates. After lysis, on the seventh day, a large portion of unmeasured soluble nitrogen compounds was liberated into the medium.

Proline is not utilized by *S. griseus* by a direct rapid deamination reaction,

since NH₄-N does not accumulate. Reductive ring rupture to delta-amino-valeric acid, shown to occur with *Clostridium sporogenes* by Strickland (1945), is not the major reaction with *S. griseus*. Neither this compound nor alpha-aminovaleric acid as the N source will support growth of *S. griseus*, either alone or combined with 0.02 per cent (NH₄)₂HPO₄ . The oxidative product, alpha-keto-delta-aminovaleric acid, shown by Blanchard, Green, Nocito, and Ratner (1944) to be the product of proline breakdown by an enzyme isolated from rat kidneys and livers, was not available for test as a supplement to the culture medium. However, a very small quantity of an ether-insoluble 2,4-dinitro-phenylhydrazone was crystallized from fermentation broths corresponding to the 5th- and 6th-day cultures of figures 2 and 3. No crystals were obtained from an old lysed culture. The crystals, after washing with water and dry-
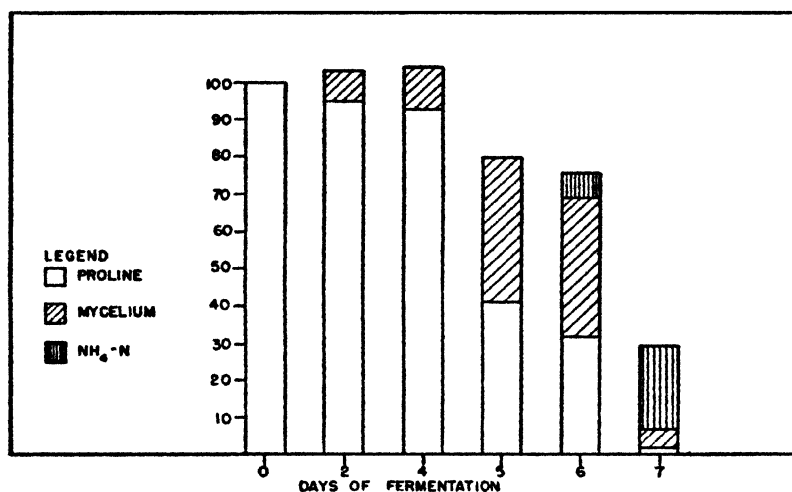
Figure 3. Nitrogen balance of *S. griseus* fermentation.

ing with ether, melted at 210 to 220 C. They resemble the 2,4-dinitrophenyl-hydrazone of alpha-keto-delta-aminovaleric acid (Krebs, 1939). Reversal of the scheme proposed for proline synthesis by *Penicillium notatum* (Bonner, 1946) does not seem probable for proline utilization by *S. griseus*.

glutamic acid → intermediate → ornithine → citrulline → arginine

proline
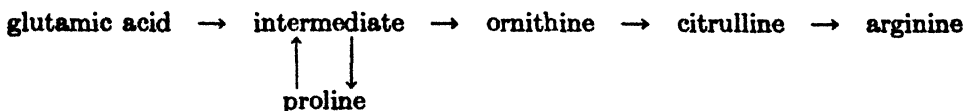
Ornithine is not used for growth. Glutamic acid, while utilized, supports only low yields of streptomycin.

Resting cell suspensions have been used to prepare the intermediate(s) in quantity. Viable *S. griseus* cells, washed well with phosphate buffer and con-centrated in volume 5 times, decomposed 0.25 mg proline per ml per hour with little NH₄-N formation.

The intermediate product produced by this method led to high yields of streptomycin by *S. griseus* when it was sterilized by filtration and added to a synthetic medium as the source of nitrogen. The intermediate is an amino-containing compound. Practically all the soluble nitrogen present at the time of maximum mycelial development may be accounted for as residual proline, NH_4-N, or amino nitrogen. The amino compound was not glutamic acid, aspartic acid, or any one of the 10 additional amino acids which may be measured by microbiological procedures (Stokes, Gunness, Dwyer, and Caswell, 1945).

DISCUSSION

Proline has a specific effect in increasing streptomycin production. Although it is interesting to speculate on proline fragments that may be combined into the streptomycin molecule as a precursor, in the manner in which the phenylacetyl radical is combined into benzylpenicillin (editorial in *Science*, 1947), the results of this investigation do not support this mechanism. Proline is utilized in the presence of NH_4-N, but streptomycin production is high only in a minimal concentration of NH_4-N or in its absence. Also, cells grown in the presence of proline are superior as an inoculum for streptomycin production to cells grown in $(NH_4)_2HPO_4$ medium. One cannot ascribe this to a precursor effect, since such cells are usually used in only 10 per cent concentration and they increase as much as ninefold on transfer to a new medium. Streptomycin accumulation, which reaches maximum rate only near the end of the cellular multiplication, is influenced greatly by the inoculum growth medium.

When proline serves as the sole source of nitrogen in a synthetic medium, there is a long lag phase of growth. This study has shown, however, that the lag phase may be reduced appreciably by supplying the culture with trace quantities of $(NH_4)_2HPO_4$. Further studies of the proline decomposition product, which accumulates before NH_4-N or streptomycin is produced, may demonstrate a less expensive nitrogen source that retains the ability to stimulate streptomycin production.

SUMMARY

Yields of 1 gram of streptomycin per liter were produced by *Streptomyces griseus* in a medium containing proline as the sole source of nitrogen. The extended fermentation time on this medium may be reduced by the addition of traces of $(NH_4)_2HPO_4$. Excess NH_4-N causes reduced streptomycin yield.

A vegetative inoculum of *S. griseus* developed on a proline medium is more satisfactory for streptomycin production than is an inoculum produced on $(NH_4)_2HPO_4$ medium. During growth on proline medium, or by the action of washed cells, *S. griseus* produces an unidentified amino-nitrogen-containing intermediate by a mechanism different from that described for other microorganisms. The intermediate acts like proline in supporting increased streptomycin production by *S. griseus*.

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STUDIES ON THE RETENTION OF HEXACHLOROPHENE (G-11) IN HUMAN SKIN

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Recent studies (Traub, Newhall, and Fuller, 1944, 1945; Seastone, 1947; Gump, 1945) on the compound hexachlorophene (G-11) (2,2'dihydroxy 3,5,6,3',5',6'hexachloro-diphenyl-methane)[1] have indicated a prolonged bacteriostatic effect on human skin. In order to explain this protracted effect, studies were conducted to determine whether hexachlorophene could be recovered from skin for several days following its application and whether the skin lipoids play any part in the retention of the material. As a control, the residual bacteriostatic effectiveness of zephiran chloride (dimethylbenzylalkylammonium chloride) was examined.

MATERIALS AND METHODS

One per cent hexachlorophene (G-11) liquid soap was prepared as follows: Ten grams of the monopotassium or monosodium salt of hexachlorophene[2] were dissolved in 50 ml of hot 95 per cent ethyl alcohol. This alcoholic solution was added with thorough mixing to 1 liter of soap solution prepared by dissolving 200 grams of clear potash soap in 800 ml of hot distilled water.

The method of determining numbers of skin organisms was essentially that of Price (1938) as modified by Pohle and Stuart (1940). The individual to be tested wet his hands to a point approximately 1 inch above the wrist joint in a liter of sterile distilled water in a sterile basin; to this wet skin area Ivory soap was applied for 25 seconds and the hands were thoroughly massaged with the lather for 75 seconds. The hands were then rinsed for 25 seconds in the liter of sterile water, and, after stirring, 1.0- and 0.1-ml samples were plated. Counts were made after incubation at 37 C for 48 hours and reported as numbers of organisms per liter of wash water. To inhibit the hexachlorophene carried over in the wash water 1 per cent sterile sheep serum was added to the agar plates. The individuals used in the tests consisted in part of laboratory personnel, the remainder being students, with men and women about equally represented. Most of the counts obtained before treatment ranged from 1 to 10 million organisms per liter of basin water.

In determining the residual hexachlorophene in the skin, the application of the agent was carried out as follows: 3 to 5 ml of the liquid soap were applied to the hands after wetting the skin with tap water; the resulting lather was massaged into the skin for 3 minutes, rinsed in tap water, and the application repeated in the same manner, giving a total contact time of 6 minutes. After a final tap

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[1] W. S. Gump, U. S. Patent no. 2,250,480.
[2] Obtained from Givaudan-Delawanna, Inc., Delawanna, New Jersey.

water rinse, the hands were dried and the residual hexachlorophene was extracted from the skin of both thumbs by inserting each thumb in turn into the same 60-ml centrifuge tube containing 15 ml of ether.   The tube was inverted for 1 minute over each thumb, with Penrose tubing connecting the thumb and the tube to prevent leakage.   The ether was then distilled off with a water-cooled condenser. Ten ml of broth were added to the residue, and the mixture was sterilized by autoclaving at 15 pounds pressure for 15 minutes.   This redissolved ether extract was diluted serially with sterile broth, and 5.0 ml were inoculated with 0.1 ml of a 24-hour broth culture of *Staphylococcus albus*, standardized turbidimetrically.   These tubes were then incubated at 37 C and read at 24-, 48-, and 72-hour intervals.   Control dilutions in broth showed that the autoclave temperature had no effect on the activity of hexachlorophene.

A series of controls in which the thumbs of 50 individuals were extracted with ether prior to contact with hexachlorophene failed to show any inhibitory effect

TABLE 1

Amount of extractable hexachlorophene at varying time intervals following three consecutive six-minute daily washes

TIME AFTER HEXACHLOROPHENE*	INHIBITORY DILUTION OF EXTRACT	AVERAGE CALCULATED AMOUNT OF HEXA- CHLOROPHENE	AVERAGE AMOUNT OF HEXACHLOROPHENE PER SQUARE INCH OF SKIN
		μg	μg
Immediately...................	1/1,000 dil.	100	8.0
24 hours.............	1/50 dil.	5	0.4
48 hours.	1/10 dil.	1	0.08
72 hours......	No inhibition	None	—
96 hours......	No inhibition	None	—

* Sum of 10 individuals per test.

in the undiluted extracts.   The amount of hexachlorophene in 5 ml of broth necessary to inhibit growth for 48 hours at 37 C of the standard inoculum of *Staphylococcus albus* was found to be 0.1 μg (0.0001 mg).   The highest inhibitory dilution of skin extract was assumed to contain this amount of hexachlorophene. The total average skin area of both thumbs was assumed to be 12 square inches. Chemical methods or the use of a spectrophotometer in quantitatively determining the amounts of hexachlorophene present in various solvents was found to be inadequate for detecting the small amounts encountered in this experimental work.

EXPERIMENTAL RESULTS

The amount of hexachlorophene deposited on the skin by three consecutive daily 6-minute washes with the agent in liquid soap was determined immediately afterward, and at 1-, 2-, 3-, and 4-day intervals following the last contact. Table 1 shows that approximately 8.0 μg of hexachlorophene were recovered from each square inch of skin immediately after the third application.   Twenty-four hours after the third application the amount recovered was 0.4 μg per square

inch, and after 48 hours 0.08 μg was found.   Residual hexachlorophene was not detectable 72 or 96 hours after the last contact with the agent.

The amount of hexachlorophene recovered when 2 per cent hexachlorophene bar soap[3] was substituted for the liquid soap tested above was determined.   The same three consecutive daily 6-minute washes were employed.   In six individuals the average amount recovered immediately after the last application was 0.8 μg per square inch of skin, or only one-tenth the amount recovered after a 1 per cent liquid soap.

A parallel series of tests with 1 per cent zephiran chloride was planned in order to determine its persistence in skin as compared to 1 per cent hexachlorophene. However, since no prolonged bacteriostatic effect was noted, further study of this material was abandoned.   Table 2 presents the results indicating the absence of any residual effect on the skin flora 24 hours after three daily 6-minute appli-

TABLE 2

Comparison of residual effects of zephiran chloride and 1 per cent hexachlorophene following three consecutive six-minute daily washes

SUB-JECTS	ORIGINAL COUNT	24 HOURS AFTER ZEPHIRAN CHLORIDE	SUB-JECTS	ORIGINAL COUNT	24 HOURS AFTER HEXA-CHLOROPHENE	SUB-JECTS	ORIGINAL COUNT	48 HOURS AFTER HEXA-CHLORO-PHENE
A	14,100,000	7,380,000	F	2,830,000	230,000	K	23,200,000	441,000
B	4,810,000	3,480,000	G	9,560,000	430,000	L	7,740,000	231,000
C	5,230,000	3,490,000	H	3,180,000	90,000	M	14,000,000	185,000
D	240,000	280,000	I	3,360,000	120,000	N	6,950,000	620,000
E	1,250,000	770,000	J	2,220,000	109,000	O	5,130,000	840,000

Figures represent the number of organisms per liter of wash water.

cations of zephiran chloride.   The hexachlorophene effect is still evident 48 hours after application.

In view of the fat solubility of hexachlorophene noted by Gump (1945), an attempt was made to determine whether its residual bacteriostatic effect was due to solution and retention in the natural fats and oils of the skin.   If this were true, the extraction of the skin lipoids before treatment with hexachlorophene should reduce the residual bacteriostatic effect.   However, the opposite effect was obtained.   The normal resident bacterial counts of six individuals were determined prior to the start of the experiment.   The hands were then immersed for 2 minutes in equal parts of acetone and ether, and this extraction was repeated in order to ensure as complete removal of fats and oils as possible, after which the hands were soaped with 1 per cent hexachlorophene soap solution for 3 minutes and rinsed, and the procedure was repeated, giving a total contact time of 6 minutes.   Twenty-four and 72 hours following this single hexachlorophene wash, skin counts were taken.

A control group of 6 individuals was tested after an *ether-acetone wash without*

[3] The solid soap was furnished through the courtesy of Johnson and Johnson, New Brunswick, New Jersey.

hexachlorophene; another group of 6 persons was studied after a single hexa-chlorophene 6-minute wash without ether-acetone pretreatment. Table 3 shows the greater and more prolonged effect of hexachlorophene on those persons whose skin had been defatted before application of the agent. The residual material recovered from the ether-acetone mixture showed no inhibitory effect upon hexachlorophene *in vitro*.

TABLE 3

Comparison of residual effect of hexachlorophene on defatted and normal skin

SUBJECTS	TREATMENT	BEFORE TREATMENT	24 HOURS AFTER WASH	72 HOURS AFTER WASH
A	Defatted with ether-acetone.	3,620,000	10,000	740,000
B	6-minute hexachlorophene	1,710,000	90,000	2,540,000
C	wash	220,000	200,000	180,000
D		11,940,000	20,000	150,000
E		2,920,000	100,000	640,000
F		2,710,000	40,000	200,000
Average..........................		3,840,000	76,600	762,000
G	Defatted with ether-acetone.	1,830,000	230,000	1,970,000
H	No hexachlorophene wash	2,440,000	1,420,000	1,550,000
I		220,000	140,000	92,000
J		4,020,000	4,720,000	3,760,000
K		2,290,000	1,130,000	2,650,000
L		3,590,000	610,000	1,190,000
Average..		2,400,000	1,370,000	1,868,000
M	6-minute hexachlorophene	8,940,000	1,750,000	1,930,000
N	wash. No ether-acetone	750,000	20,000	1,140,000
O	treatment	2,380,000	210,000	1,160,000
P		11,230,000	4,630,000	3,150,000
Q		7,340,000	1,170,000	2,220,000
R		4,330,000	123,000	120,000
Average*.........		5,830,000	1,300,000	1,620,000

The figures represent the number of organisms per liter of wash water.

* The counts shown above are higher than those in table 2 because only a single hexa-chlorophene application was used.

In a previous study (Seastone, 1947), it was noted that the bacteriostatic potency of hexachlorophene was depressed about 100-fold by the addition of 1 per cent serum to the broth or agar used for testing. A brief experiment was conducted to determine whether albumin or globulin is responsible for this effect. Sterile purified egg albumin was diluted in sterile water to a concentration comparable with that of normal serum albumin (4.8 per cent). One-tenth-ml amounts of this solution were added to 9.8-ml amounts of sterile broth containing

varied concentrations of hexachlorophene. These were inoculated with 0.1 ml of the standarized 24-hour broth suspension of *Staphylococcus albus*, incubated at 37 C, and read at 24-, 48-, and 72-hour intervals. Purified human globulin was similarly diluted to a concentration comparable with that of normal serum (1.9 per cent) and the above-described procedure was repeated. Controls on the albumin, globulin, whole serum, and hexachlorophene broth were included. It appears from table 4 that both globulin and albumin show the hexachlorophene-inhibiting effect of whole serum.

TABLE 4

Depressing effect of serum components on bacteriostatic action of hexachlorophene

Hexachlorophene..	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Control	−	−	−	+	+
1% serum	−	+	+	+	+
0.0483 % albumin . . .	−	+	+	+	+
0.0189% globulin .	−	+	+	+	+

+ = growth of standard inoculum of *Staphylococcus albus* in 72 hours.
− = no growth in 72 hours.

DISCUSSION

The prolonged bacteriostatic effect of hexachlorophene, as might be expected, is explained by retention in the skin of detectable amounts of the material during the time interval in which its effect on the skin flora is apparent. When a solid soap vehicle was substituted for a liquid, the amount of retained hexachlorophene was reduced about tenfold. This is of interest in view of a similar observation (Seastone and Erickson, 1948) based on skin counts, in which it was shown that the solid soap vehicle is also less effective in reducing the resident bacterial skin flora. The most obvious explanation for this difference lies in the fact that a much larger amount of the agent comes into contact with the skin when it is dissolved in a liquid soap vehicle.

The unexpected enhancement of bacteriostasis following defatting of the skin may indicate that a larger reservoir becomes available for the retention of hexachlorophene following this treatment. It implies that the residual hexachlorophene activity is not due to solution and retention in the lipoids of the skin.

One finding of incidental interest, not reported elsewhere in this paper, is the rare occurrence of unusually high skin counts in individuals using hexachlorophene. This has been seen only twice in our experience, the counts being several million per liter of wash water. In both cases the skin flora proved to be entirely gram-negative bacilli of the coliform group and in both cases these high counts were transitory since tests after a few days' continued use of the compound showed characteristic low counts. The phenomenon could not be induced again in these individuals, indicating that a permanent resistant flora was not established.

SUMMARY

Hexachlorophene (G-11) has been recovered from skin 2 days after three consecutive daily 6-minute applications of 1 per cent solution in liquid soap.

Approximately ten times less hexachlorophene was recovered from skin following the use of a 2 per cent preparation in solid soap.

One per cent aqueous zephiran chloride exhibited no prolonged residual bacteriostatic effect on the skin.

Preliminary treatment of skin with acetone and ether increased and prolonged the bacteriostatic effect of hexachlorophene in the skin.

The effect of whole serum in reducing the bacteriostatic effect of hexachlorophene could be duplicated by egg albumin or serum globulin.

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ANAEROBIC FORMATION OF FUMARIC ACID
BY THE MOLD RHIZOPUS NIGRICANS[1]

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Fumaric acid formation in high yields from sugar by fungi belonging to the genus *Rhizopus* has been known for a long time and studied extensively (Gottschalk, 1926; Butkewitsch and Federoff, 1929a,b, 1930a,b; Foster and Waksman, 1939a,b). The following scheme first proposed by Gottschalk epitomizes present concepts of the mechanism of fumarate formation from hexose.

$$-4H \left| \right| +4H$$

$$C_6H_{12}O_6 \longrightarrow 2CH_3COCOOH \xrightarrow{decarboxylation} 2CH_3CH_2OH + 2CO_2$$

hexose   pyruvic acid   ethyl alcohol

$$\downarrow +O$$

$$\underset{\text{fumaric acid}}{\overset{\displaystyle CHCOOH}{\underset{\displaystyle CHCOOH}{\|}}} \xrightarrow[+O]{-2H} \underset{\text{succinic acid}}{\overset{\displaystyle CH_2-COOH}{\underset{\displaystyle CH_2-COOH}{|}}} \xleftarrow[+O]{-2H} \underset{\text{acetic acid}}{2CH_3COOH}$$
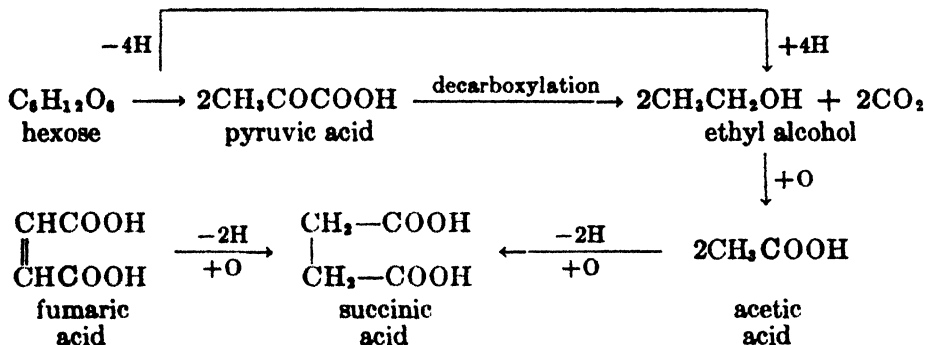
A typical alcoholic fermentation is the first stage. The alcohol (or the acetaldehyde) undergoes aerobic oxidation to acetate, this in turn being oxidized via the Thunberg-Wieland condensation yielding succinate, thence to fumarate. With an exceptionally high fumarate-yielding strain (no. 45) of *Rhizopus nigricans* we observed that the rate of conversion of alcohol into fumarate by preformed mycelium was too slow to account for its formation from glucose, indicating a possible alternative mechanism of fumarate synthesis. This has been demonstrated, and it differs from the mechanism above in that it is independent of oxygen.

EXPERIMENTAL DATA

Methodology was in general similar to that employed previously (Foster and Waksman, 1939a,b), except that fumarate was determined according to Stotz (1937), glucose by Shaffer and Somogyi's method (1933), and alcohol by dichromate oxidation. *R. nigricans* no. 45 proved distinct from other fumaric-acid-forming strains in two respects: (1) it produces the highest yields aerobically, amounting on a weight basis to 45 to 55 per cent of the sugar consumed (Foster and Waksman, 1939b); (2) fumarate formation from glucose by washed surface pads is not eliminated anaerobically, the yields under this condition ranging from

one-third to one-half of those obtainable aerobically (table 1). Calcium carbonate was present to neutralize the accumulated acidity.

The fumaric acid was isolated by ether extraction and identified by melting point, unsaturation, and neutralization equivalent. If one calculates all the products in terms of C_2 equivalents, the total is in excess of the amount of C_2 theoretically obtainable from hexose by the alcoholic fermentation mechanism. As seen from column 4 in table 1, 16.66 mM C_2 are available but a total of 19.17 mM C_2 was recovered as alcohol, lactate, and fumarate. The C_2 equivalency of the alcohol and lactate is unmistakable; the fumarate apparently causes the discrepancy and one can only conclude that it does not arise via the assumed condensation of $2C_2$.

TABLE 1

Action of Rhizopus nigricans no. 45 on glucose solution* in hydrogen atmosphere

	GLUCOSE CULTURE			WATER CONTROL
	g	*mM*	*mM C_2*	
Glucose consumed... ...	1.494	8.33	16.66	—
Fumaric acid...	.320	2.76	5.52	None
Calcium in solution†..	.130	—	—	None
Calcium accountable as fumaric acid‡. ...	.110	—	—	—
Ethyl alcohol..	.582	12.65	12.65	—
Lactic acid‡	.090	1.00	1.00	—
Volatile acids. . .	Trace	—	total: 19.17	None
Weight conversion of glucose to fumaric acid, per cent.. ..	21.4	—	—	—

* Fifty ml of 3 per cent glucose.

† No free acidity was present as judged by titration of a 5-ml aliquot. Complete neutralization of the acids in an aliquot was achieved by warming the solution before analysis.

‡ Later work showed lactic acid to be the other acidity. In this case the remaining Ca in solution was considered as Ca-lactate, i.e., 0.090 g lactic acid (=1.00 mM).

An alternative mechanism and one that could proceed independently of oxygen is the C_3 + C_1 condensation (Wood-Werkman reaction) between pyruvate and carbon dioxide to yield oxalacetate, which, by means of well-known secondary reactions, is converted to fumarate.

$$CO_2 + CH_3COCOOH \longrightarrow HOOCH_2COCOOH \xrightarrow{+2H}$$
$$\text{pyruvate} \qquad\qquad \text{oxalacetate}$$

$$HOOCCH_2CHOHCOOH \xrightarrow{-HOH} HOOCCH:CHCOOH$$
$$\text{malate}$$
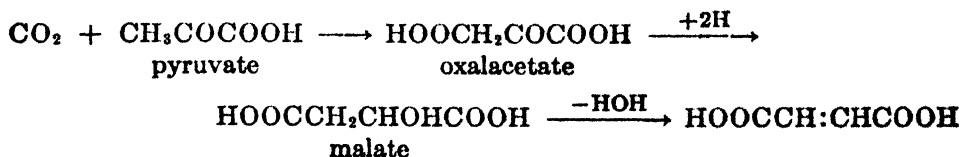
None of the evidence available is inconsistent with this premise. A C_3 origin of fumarate is in agreement with the amount available from the sugar. The

balance now becomes:

<div align="center">C$_3$ available from sugar, 16.66 mM</div>

C$_3$ equivalency of
     alcohol... 12.65 mM
     lactate... 1.00
     fumarate 2.76

          Total found............... .... 16.41

It has not been possible to demonstrate an actual uptake of CO_2 because a great excess of CO_2 is formed concomitantly during the alcohol fermentation stage. Gas exchange experiments were done in Warburg respirometers. Homogenous cell material was obtained on a shaker in 5 per cent glucose salts medium containing precipitated $CaCO_3$ as a neutralizing agent. The mycelial suspensions obtained thereby were not ideal, for they consisted of clumps and small balls of varying sizes, but this technique did allow apportionment of equal amounts of mycelium. As will be seen later, these clump structures proved to be a source of difficulty. Three-day-old mycelium was collected on a filter, dispersed in water to allow excess $CaCO_3$ to settle out, and washed in dilute (1 per cent) HCl to dissolve adhering carbonate. Evidently $CaCO_3$ is bound very tenaciously by the mycelium, for sometimes repeated or prolonged washings failed to remove all of it. It was a mistake to assume that all the carbonate was eliminated even after it appeared that no more could be shaken out in water. Following a final water wash, the mycelium was sucked free of excess moisture on a Buchner. The thin pad of mycelium disintegrates easily, and aliquots are weighed on the analytical balance. Dry weight equals about 16 per cent.

Several anaerobic flask experiments were conducted with mycelium, prepared as above, acting on glucose in desiccators with a natural gas (CH_4) atmosphere. To minimize diffusion limitations, the desiccators were set on a shaking machine. Anaerobiosis was checked periodically by placing freshly inoculated slants of *Bacillus subtilis* in the desiccator; not even the slightest visible growth was ever observed on these slants indicating absence of oxygen. Decolorized methylene blue glucose solution also was used as an indicator of anaerobiosis. An alcohol solution was supplied to control mycelium in each desiccator as a check against fumarate formation via C$_2$ condensation. This was never observed.

Age of mycelium. For both shaker or surface culture, the age of the mycelium influences its capacity to form fumarate from glucose anaerobically. Thus, 1-, 2-, and 3-day-old shaker mycelium produced, respectively, 10.3, 13.1, and 17.4 per cent weight conversion of glucose to fumarate. The effect was much more marked with surface mycelium: 5-, 7-, and 9-day-old mycelium gave conversions of 0.6, 3.3, and 18.1 per cent, respectively.

Effect of pH. Mycelium harvested from a neutral medium is superior in anaerobic fumarate formation to mycelium from acid media. But, in the anaerobic replacement phase, both neutral- and acid-grown mycelium require acid conditions for maximum fumarate formation (table 2). Neutrality was maintained

by CaCO₃. Treatments with no CaCO₃ rapidly become acid. The yield in acid environment was 3 to 4 times that under neutral conditions (excess CaCO₃).

Excessively low pH values (below pH 2.5) are inhibitory to further accumulation of fumarate. The pH drop to 2.5 in the acid treatments is due solely to the organic acidity resulting from fermentation of the glucose. Neutral-grown mycelium always traps a small amount of carbonate, which prevents the pH from falling to an inhibitory level in the replacement phase when no added neutralizing agent is present. The optimum pH range is about 3.0 to 4.0. When sufficient carbonate is added to maintain the pH significantly above 4.0, there is a sharp reduction in fumarate yield. Thus CaCO₃ added at the rate of 0.1 to 0.4 mg per ml maintained the pH between 3.4 and 3.8 with a maximum conversion yield of fumarate of 14.5 per cent.

One mg CaCO₃ per ml maintained the pH at 4.75, resulting in only one-half the maximum fumarate yield. This pH effect is emphasized here because it

TABLE 2
Acidity and anaerobic fumarate formation*

TYPE OF MYCELIUM	REPLACEMENT PHASE	FINAL pH	FUMARIC ACID FORMED	WT. CONVERSION OF GLUCOSE TO FUMARIC
			mg/ml	*per cent*
Acid	Acid	2.5	2.98	8.7
Acid	Neutral	6.4	1.11	3.2
Neutral	Acid	3.7	4.20	12.3
Neutral	Neutral	6.5	1.07	3.1
Neutral ⎫ †	Acid	0	—	
Control ⎭	Neutral	0	—	

* Glucose consumed in each case was 34.2 mg per ml. Incubation, 2 days.
† Water only in these treatments.

underscores the difference in the optimum pH for the formation of fumarate by *R. nigricans* aerobically and anaerobically, additional evidence for two different mechanisms in this organism. Aerobically excess carbonate (i.e., nearly neutral pH) is optimum where highest yields are crucial (Kane *et al.*, 1944; Waksman, 1944).

The fact that an acid range was optimum for anaerobic fumarate formation facilitated greatly subsequent experimental work, for it was possible to dispense with aseptic precautions in handling the mycelium and solutions because of the rapid development of acidity, which effectively prevents bacterial contamination.

Mycelium cultivated in phosphate-buffered medium was definitely inferior to that from CaCO₃-buffered medium with respect to anaerobic fumarate formation.

Storage of mycelium. . The storage of washed mycelium in water suspension in an icebox results in rapid loss of anaerobic fumarate-producing ability within a week. When the mycelium is held in its original growth culture fluid, however, its activity is maintained over a week or more.

Attempts to produce biotin-deficient mycelium. Direct proof of the origin of anaerobic fumarate via CO₂ fixation would be obtained if this reaction could be specifically inhibited. At present there are no poisons specific in this respect,

and the only tool available is to secure a deficiency in biotin, the coenzyme of the fixation reaction (Potter and Elvehjem, 1948; Shive and Rogers, 1947). This problem is not so simple in *R. nigricans* as it is in certain other systems because the fungus is prototrophic with respect to biotin, whereas the other systems require exogenous biotin. Three different approaches all were uniformly negative: (a) Storage of mycelium at pH 4.5 according to the method of Lichstein and Umbreit (1947), whereby *Escherichia coli* can be rendered biotin-deficient by enzymatic destruction of the cellular biotin. (b) Growth in the presence of analogues of desthiobiotin (Rogers and Shive, 1947). The following desthiobiotin analogues[2] known to be effective in certain bacteria were ineffective when tested at 1 mg per ml with very small inocula of spores or vegetative mycelium of *R. nigricans* no. 45: 4 methyl-5-(w-carboxyoctyl)-2-imidazolidone, 4 methyl-5-(w-carboxyamyl)-2-imidazolidone, and 5-(w-carboxyamyl)-2-imidazolidone. (c) Biotinless mutants. Notwithstanding the fact that *R. nigricans* sporangiospores are multinucleated (3 to 5 average) and that no segregation of nuclei is possible via sexual reproduction, an attempt was made to obtain mutants using a nitrogen mustard according to techniques especially designed to predispose the best chances for asexual segregation. Some 2,000 sporangiospores were tested[3] in complete and in minimal synthetic media without revealing a single deficiency mutant. About a dozen distinctive morphological mutants were obtained and several slow growers, which on the second or third transfer grew at the normal rate.

CO$_2$ deprivation. Another possible way of repressing specifically the carboxylation of pyruvate is to reduce the CO$_2$ concentration to a point where the fixation enzyme is unsaturated with respect to this particular reactant. Though the solubility of CO$_2$ is negligible in the pH range 3 to 4, which is optimum for anaerobic fumarate formation, it has not been possible to achieve a CO$_2$ deficiency because the mycelium vigorously generates CO$_2$ by means of alcoholic fermentation. This can only mean that the interior of the fungus cells contains significant amounts of CO$_2$ at all times, enough to keep the fixation reaction going at an undiminished rate. This was true even when the fermentation was conducted in a high vacuum, to remove intracellular CO$_2$ as quickly as possible. The vigor of the CO$_2$ formation was evidenced by a continuous stream of bubbles emanating from the hyphae kept in a vacuum. Though evacuation did remove some of the CO$_2$, there could be three reasons why it did not remove enough to suppress CO$_2$ fixation: (1) the cell sap probably is well buffered near neutrality, which means that considerable CO$_2$ would be retained in solution as bicarbonate; (2) loss of CO$_2$ from the cells is impeded by the cell membrane and wall; (3) the cells of this coenocytic organism are thick. It is questionable that CO$_2$ could be removed instantly and completely from the interior of hyphae of such large diameter. We assume reassimilation occurs before the CO$_2$ has a chance to diffuse out of these large fungus cells.

Manometric studies of gas balance. For every mole of alcohol formed there should be formed 1 mole of CO_2. Any reutilization of the latter in the synthesis of C_4 dicarboxylic acids should result in a CO_2:alcohol ratio of less than 1. Several Warburg manometric experiments showed peculiarly wide deviations in the CO_2:alcohol ratio. Only in a few cases were the ratios less than unity and most of these only slightly less. Frequently the ratio exceeded unity—and to varying degrees. In order to get sufficient fumarate formed for its determination in the manometer vessel contents, it was essential to use 3 per cent glucose solution; mercury was used as the manometer fluid. About 70 mg mycelium, moist weight, were used per vessel, the experiments generally requiring about 12 hours. The manometric studies were completed using HCl-washed mycelium before the significance of the difficulties of removing residual-trapped $CaCO_3$ was fully appreciated. We interpret the varying high CO_2:alcohol ratios obtained as being

TABLE 3

Fermentation balance of Rhizopus nigricans no. 45

	mg/ml	mmols
Glucose consumed	23.14	0.129
Fumaric acid...	3.23	0.028
Ethyl alcohol.. . .	8.55	0.186
Lactic acid*.......	4.0	0.044
CO_2	7.90	0.180
Carbon recovery....	103.3 per cent	
O/R index..	1.12	
C_3 available from glucose.......	0.258 mmols	
C_3 found in products....	0.258 mmols	

* By the method of Barker and Summerson (1941).

due to dissolution of this carbonate by the acids formed, giving that much excess CO_2. The variation in results is in accord with the differing amounts of trapped carbonate that would be expected. In some experiments, however, this source of difficulty did not obscure the nature of the events taking place. For example, in one case in which the fumarate yield from glucose was 8.2 per cent there were formed 0.0845 mM CO_2 and 0.0913 mM ethanol. The ratio of 0.92 in this case indicates reutilization of CO_2. In one case "acid" mycelium was used, that is, a limited amount of carbonate was furnished in the growth medium, and the mycelium was used 1 day after visible carbonate was dissolved. Ostensibly this mycelium was carbonate-free, and a manometric experiment in duplicate clearly showed a substantial CO_2 deficiency. The ratio was 0.81; the fumarate yield was 4.9 per cent. As noted earlier, "acid" mycelium is inferior in anaerobic fumarate formation, as is mycelium from medium buffered with phosphate. Hence the use of mycelium from carbonate-buffered media.

Table 3 gives the balance of products in another manometric experiment. The CO_2:alcohol ratio here is 0.97, but the data show this to be artificially high, because of extraneous CO_2 from residual carbonate. The C_3 balance happens to be theoretical (0.258:0.258 = 100 per cent). However, both the carbon recovery and the O/R index of the fermentation products are in excess of theory;

and in view of the theoretical C_3 balance, extra oxidized carbon must be present, i.e., CO_2 from trapped carbonate. Thus, the actual CO_2:alcohol ratio must be appreciably less than unity.

Oxalacetate decarboxylase. The presence of the enzyme responsible for CO_2 fixation has been demonstrated. The reverse action was studied, namely, the decarboxylation of oxalacetic acid (OAA). The decomposition was linear for the 1-hour duration of the experiment (table 4). Mycelium desiccated *in vacuo* over P_2O_5 was considerably more active in decarboxylation of OAA than was fresh mycelium of corresponding dry weight, presumably a permeability matter (Krampitz and Werkman, 1941). Cell material dehydrated with acetone-ether or with dioxane was inactive.

The enzymatic decarboxylation was calculated by deducting the endogenous and the boiled cell controls from the total obtained with unboiled material, and it probably represents the minimum value, because cell extractives in the boiled control contain substances that catalyze spontaneous decomposition of OAA.

In addition to all the foregoing evidence for origin of the anaerobic fumarate via CO_2 fixation in *R. nigricans* there is to be considered the direct evidence resulting from the use of radioactive carbon as a tracer (Foster *et al.*, 1941), when the carbon resided exclusively in the carboxyl groups of the fumarate. However, the tracer experiments merely indicated the qualitative nature of the reaction. There was no indication of a bulk fixation taking place, as the foregoing has demonstrated. Fumarate is formed anaerobically also by animal tissue and by *Escherichia coli* (Krebs and Eggleston, 1940), but not in bulk as above.

Based on the findings above the fermentation of glucose by *Rhizopus nigricans* no. 45 may be represented as follows:

$$nC_6H_{12}O_6$$

$$
\begin{array}{ccc}
& \downarrow +2H & \quad \downarrow -n4H \\
CH_3CHOHCOOH & \longleftarrow nCH_3COCOOH & \longrightarrow CO_2 + CH_3CHO \\
\text{lactic acid} & \text{pyruvic acid} & \text{acetaldehyde}
\end{array}
$$

$$\downarrow +CO_2$$

$$HOOCCH_2COCOOH$$
$$\text{oxalacetic acid}$$

$$\downarrow +2H$$

$$CH_3CH_2OH$$
$$\text{ethyl alcohol}$$

$$+2H \downarrow$$

$$HOOCCH_2CHOHCOOH$$
$$\text{malic acid}$$

$$\downarrow -HOH$$

$$HOOCCH:CHCOOH$$
$$\text{fumaric acid}$$
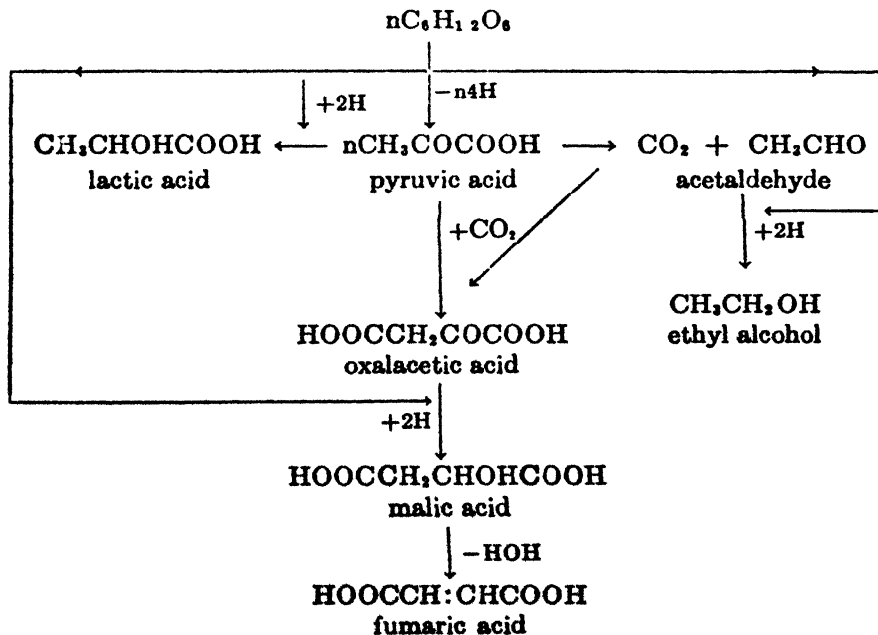
In essence this scheme represents a reduction of pyruvic acid in three ways: before decarboxylation (lactate), after decarboxylation (alcohol), and after carboxylation (fumarate via malate).

DISCUSSION

The discovery that bulk formation of a C_4 dicarboxylic acid from sugar by fungi can take place independent of oxygen and that it arises via fixation of carbon dioxide has important implications for our understanding of the carbohydrate metabolism of fungi in general, and in particular for those typified by the formation and accumulation of organic acids. It offers clarification of a number of seemingly anomalous results in the literature dealing with fumaric acid forming fungi of the *Rhizopus nigricans* type and also citric acid formation by *Aspergillus niger* strains (Wang, 1941; Barinova, 1941; Butkewitsch and Gaevskaya, 1935; Gudlet 1935a,b; Zhuravskiĭ, 1939; Wells, Moyer, and May, 1936).

TABLE 4

Oxalacetate decarboxylase in Rhizopus nigricans mycelium

TREATMENT	CO_2 IN 1 HR
	μl
30 mg dry wt. mold, no OAA ..	126
30 mg dry wt. mold + 0.01 mM OAA........	377
30 mg dry wt. mold boiled, + 0.01 mM OAA	58
CO_2 due to enzymatic decarboxylation........	193

The anomalies arise from the fact that physiological balances between carbohydrate consumed and CO_2 and organic acids produced were not consistent with values expected according to the mechanisms conventionally used to interpret their formation. These mechanisms all predicate an initial alcoholic fermentation and subsequent aerobic condensation of the C_2 moieties to C_4 and, in the case of citrate, of $C_4 + C_2$. Because of acid yields exceeding those possible by these schemes, mechanisms such as a direct $C_4 + C_2$ split of the hexose chain and even a closing of the C_6 chain have been postulated.

These novel theories do not have to be resorted to if one considers that all the available data can be reconciled to the fact that in various fungi two mechanisms for the synthesis of C_4 decarboxylic acid can take place concomitantly, namely, $C_2 + C_2$ and $C_3 + C_1$. The C_1 is reutilized as a by-product of the C_4 formation. This is a logical explanation for the high yields of fumarate and citrate and the low CO_2 yields reported for certain strains. This idea was suggested on the basis of qualitative CO_2 fixation studies with C^{11} (Foster *et al.*, 1941) in which the fixed CO_2 was located in the carboxyl groups of fumarate and citrate. The bulk formation of C_4 reported now lends added weight to this idea.

Special note must be made of the fact that four other strains of *Mucorales* that produce fumaric acid aerobically do not produce fumarate anaerobically. This

affords one explanation of strain specificity with regard to organic acid production by fungi, at least for fumarate and citrate, and possibly for others. That is, strain specificity, in part at least, depends on the possession of a bulk CO_2 fixation mechanism that supplements the regular C_2 condensation mechanism for synthesis of C_4 and C_6 acids. Different strains may possess this mechanism to varying degrees. Doubtless all exceptionally high yielding strains will be found to possess a bulk CO_2 fixation mechanism. In the case of citric acid, it is well to keep in mind the possibility of bulk CO_2 fixation via oxalosuccinic acid.

There is reason to believe a similar situation holds for the formation of oxalic, itaconic, and succinic acids by fungi.

SUMMARY

A strain of *Rhizopus nigricans* (no. 45) forms fumaric acid anaerobically from glucose in yields approximating 20 per cent of the carbohydrate consumed. The other fermentation products are ethyl alcohol, lactic acid, and CO_2. Evidence is presented that indicates that the fumarate is formed by CO_2 fixation and involves the Wood-Werkman reaction (pyruvate $+ CO_2$). Various factors influencing this fermentation are reported. The fungus mycelium is shown to contain the enzyme that is responsible for CO_2 fixation, oxalacetate decarboxylase. This organism possesses two mechanisms for fumarate formation: (1) the above-described $C_3 + C_1$ fixation, which can proceed anaerobically; (2) a $C_2 + C_2$ condensation that occurs only aerobically. Probably both reactions proceed concomitantly in the presence of air. Existence of the bulk $C_3 + C_1$ mechanism offers an explanation of the yields of organic acids (fumaric, citric, and oxalic) formed by fungi in excess of those compatible with an origin exclusively from C_2 intermediates.

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EVALUATION OF PRECURSORS FOR PENICILLIN G[1]

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During the war, it was found that phenylacetic acid was a constitutent of penicillin G. Hence phenylacetic acid and its derivatives were used in culture media for penicillin production in English and American laboratories. Publication of the work was delayed because of wartime restrictions. In early work on precursor addition *Penicillium notatum* NRRL 1249.B21 was used in surface cultures, and *P. notatum* NRRL 832 and *Penicillium chrysogenum* 1951.B25 in submerged cultures (Moyer and Coghill, 1947; Glaxo Laboratories, Ltd., *et al.*, 1940; Coghill and Moyer, 1947). It may be pointed out here that workers in this field have so far been able to modify only the side chain of the penicillin molecule. Even in this respect no success has been achieved in inducing the biosynthesis of penicillins with aliphatic side chains (Stone *et al.*, 1946; Smith and Bide, 1948).

Most work has been done on precursors for penicillin G, because of its greater therapeutic value (Clowes and Keltch, 1947; Turner *et al.*, 1947; Eagle and Fleishman, 1948). The Editorial Board of the Monograph on the Chemistry of Penicillin (1947) has recently published a list of representative compounds effective as precursors for penicillin G, the data for which were obtained from Glaxo, Lilly, Cutter, Heyden, and Abbott Laboratories.

In this study an attempt has been made to determine the effect of various types of penicillin G precursors on penicillin yield, proportion of penicillin G, time of fermentation, and pH.

EXPERIMENTAL METHODS

Fermentation techniques. *P. chrysogenum* Q176 was used exclusively in this study. Data regarding the origin of this strain and regarding the penicillin yields and types obtained by its use have already been published (Backus *et al.*, 1946; Higuchi *et al.*, 1946; Jarvis and Johnson, 1947; Winsten and Spark, 1947).

Vegetative inoculum prepared by the method described by Gaily *et al.* (1946) and Jarvis and Johnson (1947) was used throughout. It was found convenient to dilute the inoculum with an equal amount of sterilized distilled water to increase its fluidity.

The fermentation medium contained (in grams per liter): lactose 30.0, glucose 10.0, ammonium acetate 3.5, ammonium lactate 5.5, and the standard salt mix-

ture of Jarvis and Johnson (1947). Ammonium lactate was prepared by adjusting diluted and depolymerized lactic acid to pH 8.6 with ammonium hydroxide. In fermentations with β-phenylethylamine as precursor, the concentration of ammonium lactate was increased to 6.0 grams per liter. The sugar solution was sterilized separately and added to the medium just before inoculation. The final pH of the basal medium was always between 6.0 and 6.2. This synthetic medium was used in all fermentations except those of figure 5b, in which a corn steep liquor medium was employed. This contained (in grams per liter): corn steep liquor (dry basis) 30, lactose monohydrate 30, glucose 10, and calcium carbonate 10.

Eighty ml of fermentation medium were incubated in 500-ml Erlenmeyer flasks. The medium consisted of 47 ml salt solution, 25 ml sugar solution, and 8 ml diluted inoculum. The flasks were incubated at 23 C on a rotary shaker, which described a 1-inch circle at 320 to 330 rpm. Samples for penicillin assays were taken daily under aseptic conditions.

Preparation of precursors. Esters of ethyl, isopropyl, butyl, isobutyl, and ocatadecyl alcohols and of ethylene glycol were prepared by refluxing with phenylacetyl chloride in the presence of pyridine. The saponification equivalents of these esters after purification were found to be correct within ± 2 per cent.

Phenylacetylglycine and phenylacetyl-DL-alanine were prepared by the method of Hotter (1888) and decolorized with activated charcoal. Phenylacetyl-β-phenylethylamine was prepared by the method of Pictet and Kay (1909). Phenylacetylmethylamine was prepared by reacting a 5 per cent aqueous solution of methylamine with phenylacetyl chloride in an ice bath in the presence of 20 per cent NaOH. It was dried and crystallized from benzene.

The melting points found for phenylacetylglycine (143 C) and phenylacetyl-β-phenylethylamine (93 C) agreed very closely with those given in the literature. Phenylacetyl-DL-alanine and phenylacetylmethylamine melted at 141 C and 59 C, respectively.

All the precursors were sterilized separately. β-Phenylethylamine was added as the lactate at pH 6.0. Phenylacetic acid, phenylacetylglycine, phenylacetyl-DL-alanine, and DL-phenylalanine were added as aqueous solutions of their sodium salts. The pH of the phenylacetate was about neutral, that of phenylacetylglycine and phenylacetyl-DL-alanine salts was 6.0 to avoid hydrolysis during sterilization, and that of DL-phenylalanine was 10.0 to render the latter soluble. When DL-phenylalanine was used as a precursor, the pH of the contents of the flasks was adjusted to that of the control by the addition of sterile 1 N H_2SO_4. Phenylacetonitrile, β-phenylethyl alcohol, and the phenylacetates of ethyl, isopropyl, butyl, and isobutyl alcohols and of ethylene glycol were added as pure liquids, whereas phenylacetaldehyde was added as a 50 per cent solution in alcohol. Phenylacetamide, phenylacetylmethylamine, phenylacetyl-β-phenylethylamine, and octadecanol phenylacetate were added as solid compounds. At least 3 replicates of all fermentations were run. The penicillin values reported are average of these replicates.

Analytical procedures. Penicillin assays were made by the Oxford cup method

with the use of *Staphylococcus aureus* H as the test organism (Foster and Wood-ruff, 1944; Schmidt and Moyer, 1944). Pure penicillin G was used as a standard.

The pH of the samples was determined by means of a glass electrode, immediately after removal from the fermentation flasks. A delay of half an hour would cause an increase in the pH reading of 0.2 to 0.3 units because of the escape of dissolved CO_2. Penicillin G determinations were made by the method of Thorn and Johnson (1948). Free phenylacetic acid was determined by the method of Higuchi and Peterson, based on two-phase chromatography, the details of which will be published elsewhere.

RESULTS AND DISCUSSION

Toxicity of β-phenylethylamine and phenylacetic acid. A comparison of the toxic effects of β-phenylethylamine and phenylacetic acid is given in figures 1 and 2. It will be seen from figure 1 that β-phenylethylamine was effective in increasing the penicillin yield when it was added at the time of inoculation or up to 15 hours after the addition of the inoculum, when the pH was about 5.9 to 6.2. It proved to be toxic, however, when it was added at a point when the pH had risen beyond neutrality. This toxicity of β-phenylethylamine was not lessened by purification of the compound through a Fenske distillation column containing 10 to 12 theoretical plates. That the toxicity is not due to an alkaline pH alone is evident from figure 1e, indicating that practically no penicillin was produced when 0.12 per cent β-phenylethylamine was added at 24 hours and the pH during the entire fermentation remained below 8.0. In another experiment 0.60 per cent β-phenylethylamine was found to be toxic when added at the time of inoculation (table 5).

Quite the reverse was true when phenylacetic acid was used as precursor (figure 2). Phenylacetic acid was very toxic when added in high concentrations at an acid pH (figure 2c). This is in agreement with the findings of Moyer and Coghill (1947). The same amount when added at an alkaline or neutral pH (figure 2f) gave a significant increase in yield over that of the control, but the yield was much lower than that obtained with a smaller concentration (figure 2e). When lower concentrations of phenylacetic acid were added, no toxic effect was encountered, even when the addition was made at an acid pH (figure 2b).

Precursor efficiency of β-phenylethylamine. In figure 3 are shown the yields, percentage of penicillin G, and pH obtained when β-phenylethylamine was used as a precursor. No penicillin G was detected when no precursor was used. Higuchi *et al.* (1946) and Higuchi and Peterson (1947) reported the presence of 13 to 44 per cent penicillin G in the broths of *P. chrysogenum* Q176 in synthetic medium by using a differential assay method. The discrepancy is due to the fact that their method is applicable only to a mixture of three known penicillins. Since culture Q176 has been found to produce a number of known and unknown penicillins, their results on the complex mixture do not appear to be valid.

A probable explanation of this behavior is that culture Q176 is incapable of synthesizing the R group of penicillin G. Hence if a penicillin G precursor is supplied in the absence of other precursors in the initial stages, the culture uses

the precursor to produce almost entirely penicillin G.   But as the fermentation progresses, other precursors are produced with a simultaneous fall in the concentration of penicillin G precursor.   These precursors compete with the penicillin G precursor, resulting in the observed drop in the proportion of penicillin G (figure
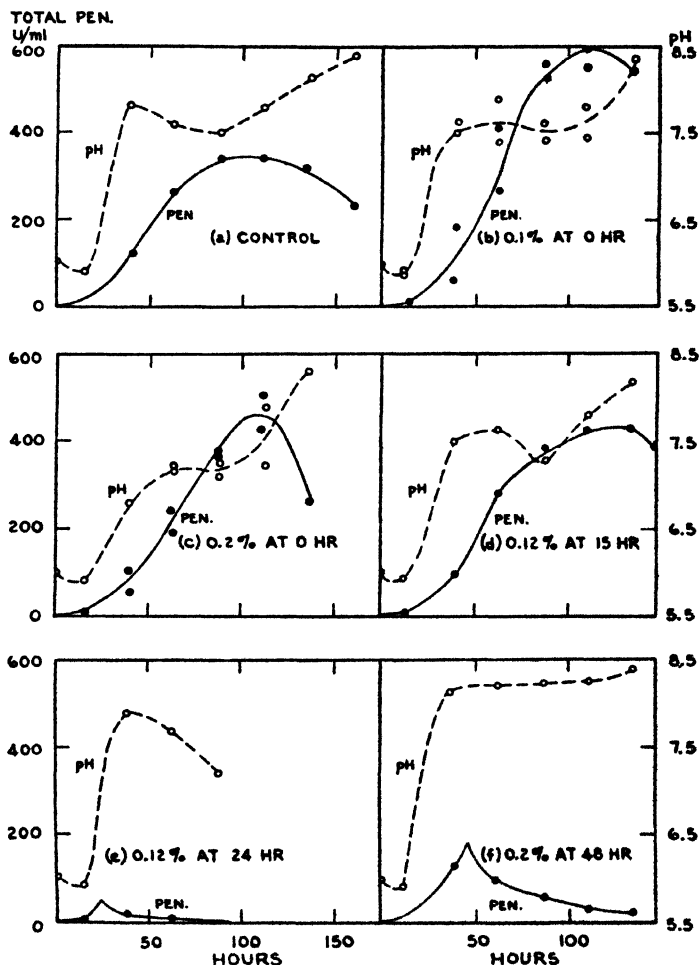
Figure 1. Effect of concentration and time of addition of β-phenylethylamine on penicillin yield and pH.   The figures for the control are the average of 5 separate runs.   The time of precursor addition and the amount added are indicated in each graph.

3c, d).   If a high concentration of penicillin G precursor is maintained by addition at intervals (figure 3e, f), utilization of endogenous precursors is suppressed; hence very little formation of other penicillins takes place.

Precursor efficiency of phenylacetic acid.   Phenylacetic acid appears to be a more efficient precursor than β-phenylethylamine (figure 4).   Not only were the yields higher, but the proportion of penicillin G was practically constant through-

out the fermentation, whether the precursor was added at once or in many equal portions. It appears that the rate of depletion of phenylacetic acid is much slower than that of β-phenylethylamine, so that a better supply is always available.
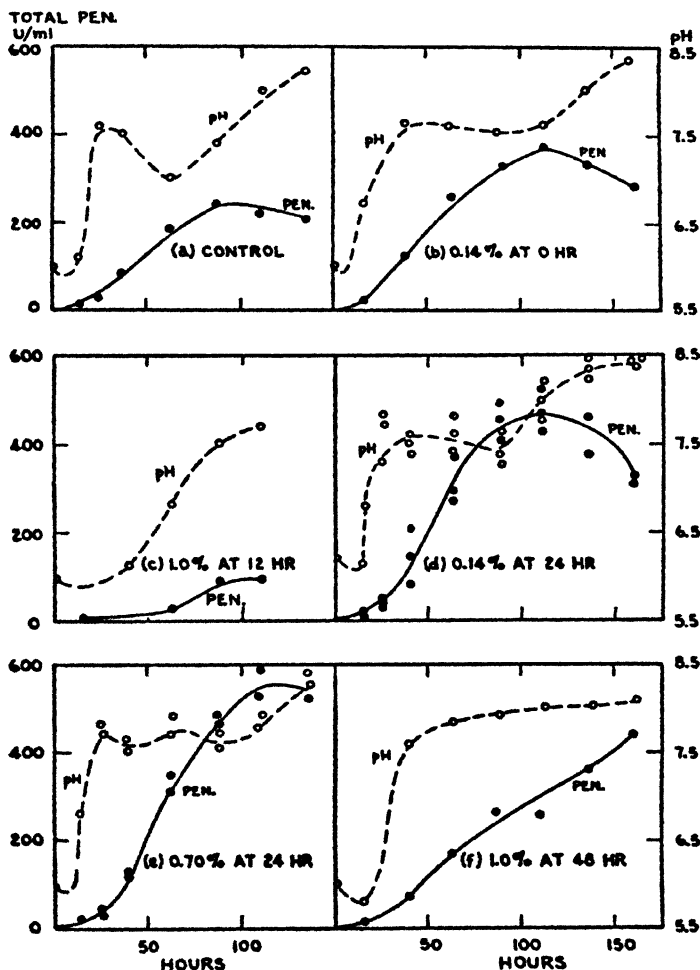
Figure 2. Effect of concentration and time of addition of phenylacetic acid on penicillin yield and pH. The figures for the control are the average of 13 separate runs. The time of precursor addition and the amount added are indicated in each graph.

Optimum levels of phenylacetic acid. The data so far discussed have shown that maximum precursor efficiency is attained when the precursor addition is made at intervals in small portions. Further, phenylacetic acid has been found to be superior to β-phenylethylamine in yielding higher total penicillin and a higher proportion of penicillin G. Hence, in later experiments, phenylacetic acid was used unless otherwise stated.

To determine the optimum concentration of phenylacetic acid that could be added at approximately 12-hour intervals, a number of fermentations were run with both synthetic and steep liquor media with various levels of precursor ranging from 0.0025 to 0.50 per cent. Loss due to evaporation during the incubation
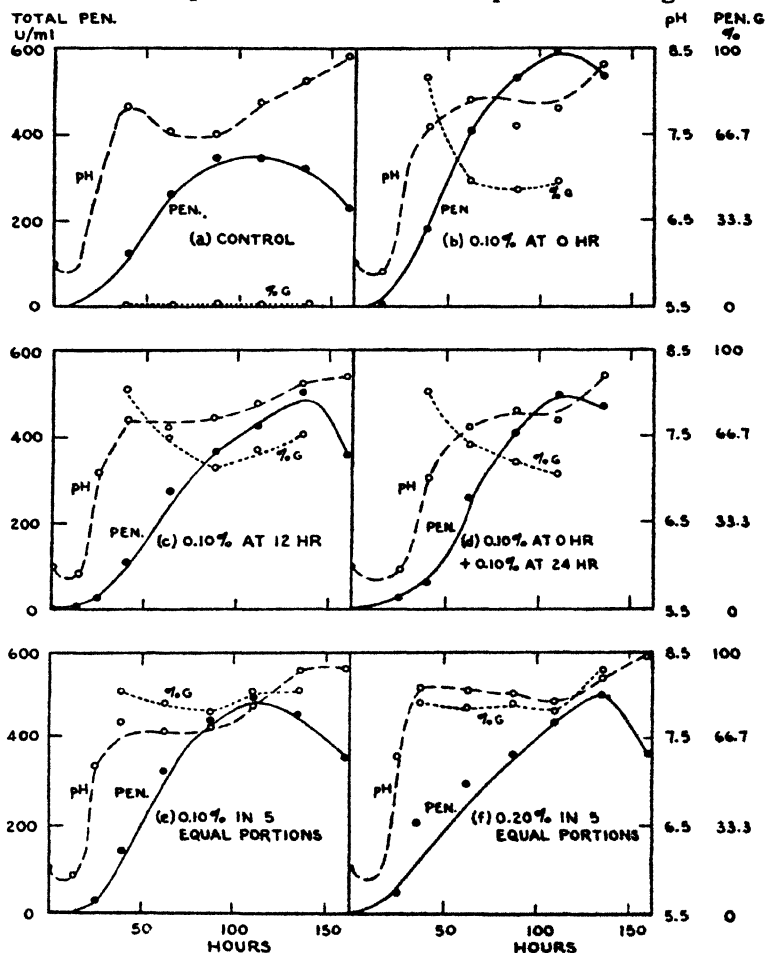
Figure 3. Precursor efficiency of β-phenylethylamine. The times of precursor addition and the amounts added are indicated in each graph. In e and f additions were made in 5 equal portions at 12-hour intervals, beginning at 15 hours.

on the shaker, determined in a number of experiments, was found to be approximately 1 ml per day. Losses from evaporation and sampling were taken into consideration in calculating the amount of precursor to be added. The data obtained in a typical experiment by using 0.02, 0.05, and 0.10 per cent phenylacetic acid every 12 hours (9 additions), beginning at the 15th hour, are shown in table 1. In general, the penicillin yields in replicate flasks check well.

The data obtained by using various precursor levels are summarized in figure

5. In the figure, data for all runs made are plotted except for the control without precursor and those runs in which the precursor addition level was zero or 0.10 per cent. Here the number of runs was so large (12 on synthetic medium and 6 on steep liquor medium) that only the average could conveniently be plotted.

From figure 5a, it will be seen that the proportion of penicillin G increased from zero in the absence of precursor to about 90 per cent when 0.02 to 0.05 per cent phenylacetic acid was added at 12-hour intervals. Though the proportion of penicillin G was always more than 90 per cent when 0.05 per cent or more pre-
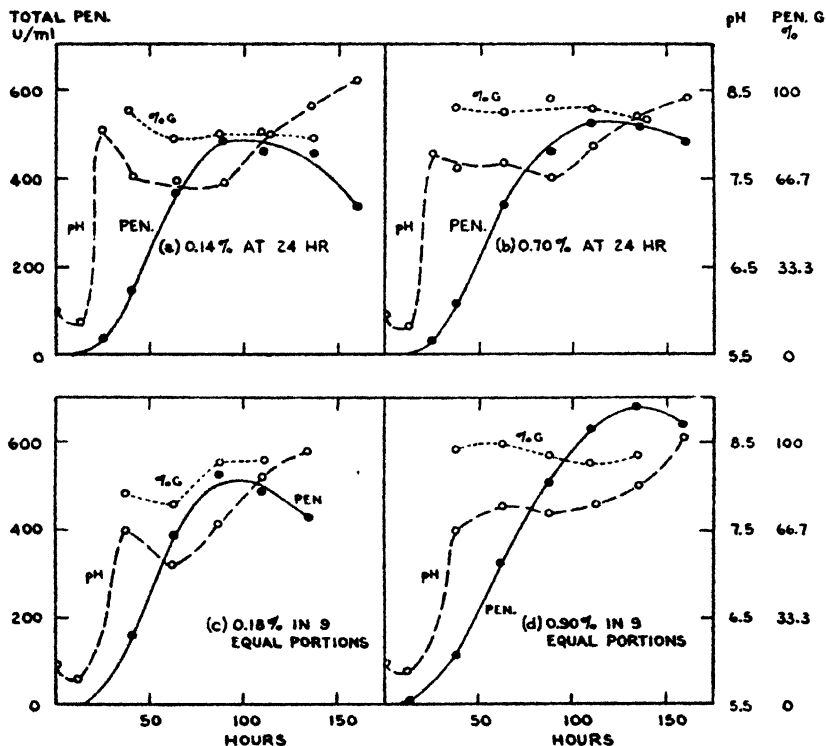
Figure 4. Precursor efficiency of phenylacetic acid. The times of precursor addition and amounts added are indicated in each graph. In c and d additions were made in 9 equal portions at 12-hour intervals, beginning at 15 hours.

cursor was used, optimal yields were only obtained when 0.10 to 0.15 per cent phenylacetic acid was added every 12 hours. The time of fermentation, however, was prolonged, presumably because of the deleterious effect of high phenylacetate concentrations on the growth of mycelium (Jarvis and Johnson, 1947). Precursor addition levels up to 0.30 per cent did not affect the average pH of the fermentation. But when 0.50 per cent or more phenylacetic acid was added every 12 hours, the pH remained below 6.6 and the yields were very low.

The foregoing picture, in general, holds for the steep liquor medium (figure 5b) with the following exceptions: (1) There was about 5 per cent penicillin G even

when no phenylacetic acid was added. (2) The penicillin yields obtained were 5 to 20 per cent higher than those obtained in the synthetic medium under identical conditions. (3) The proportion of penicillin G reached over 90 per cent only when 0.10 per cent or more precursor was added every 12 hours. (4) The average pH during the fermentation was always higher than 8.0, but it was always a little below 8.0 in the synthetic medium.

TABLE 1

A typical experiment showing variation in penicillin yield and pH in individual flasks

PHENYLACETIC ACID*	FLASK NO.	pH† AT HR					PENICILLIN U/ML AT HR				
		63	87	111	135	159	63	87	111	135	159
per cent											
A. Control	1	6.0	7.8	8.3	8.6	8.5	172	234	210	194	—
without pre-	2	6.0	7.7	8.7	8.5	8.5	200	246	216	196	—
cursor	3	6.2	7.7	8.2	8.5	8.4	208	243	240	214	—
	4	6.3	7.6	8.3	8.6	8.5	192	225	219	184	—
Average...							197	237	221	197	—
B. 0.02	1	7.4	7.8	8.2	8.6	8.8	330	440	375	368	—
	2	7.2	7.4	7.7	8.3	8.4	351	600	565	492	—
	3	7.0	7.7	8.2	8.4	8.5	492	600	500	448	—
	4	6.7	7.7	8.2	8.5	8.7	396	500	490	400	—
Average...							392	535	483	427	—
C. 0.05	1	6.9	7.8	8.2	8.5	8.8	582	606	550	476	424
	2	7.0	7.6	8.1	8.4	8.8	450	550	505	580	520
	3	6.9	7.8	8.2	8.5	8.9	462	544	625	496	368
Average...							498	567	560	517	437
D. 0.10	1	7.5	7.7	7.8	8.3	8.5	372	600	738	700	640
	2	7.5	7.7	7.7	8.2	8.4	351	615	750	825	740
	3	7.5	7.7	7.7	8.2	8.8	333	630	763	844	740
Average...							352	615	750	790	707

* Added every 12 hours (9 additions).
† The initial pH of the sterilized medium was 6.1.

Some of these exceptions can be explained on the basis that corn steep liquor contains some precursors (Mead and Stack, 1948). The presence of β-phenylethylamine is responsible for the occurrence of penicillin G even when no penicillin G precursor is added. The presence of precursors of penicillins other than penicillin G may account for the fact that comparatively higher amounts of phenylacetic acid are required to obtain high penicillin G yields.

Adaptation of the mold to phenylacetic acid. In a further effort to increase the penicillin yield, the mold was adapted by growing the inoculum in media supple-

mented with 0.02 and 0.10 per cent phenylacetic acid.   A control run was also
made simultaneously.   No difference in growth of inoculum was noticeable and
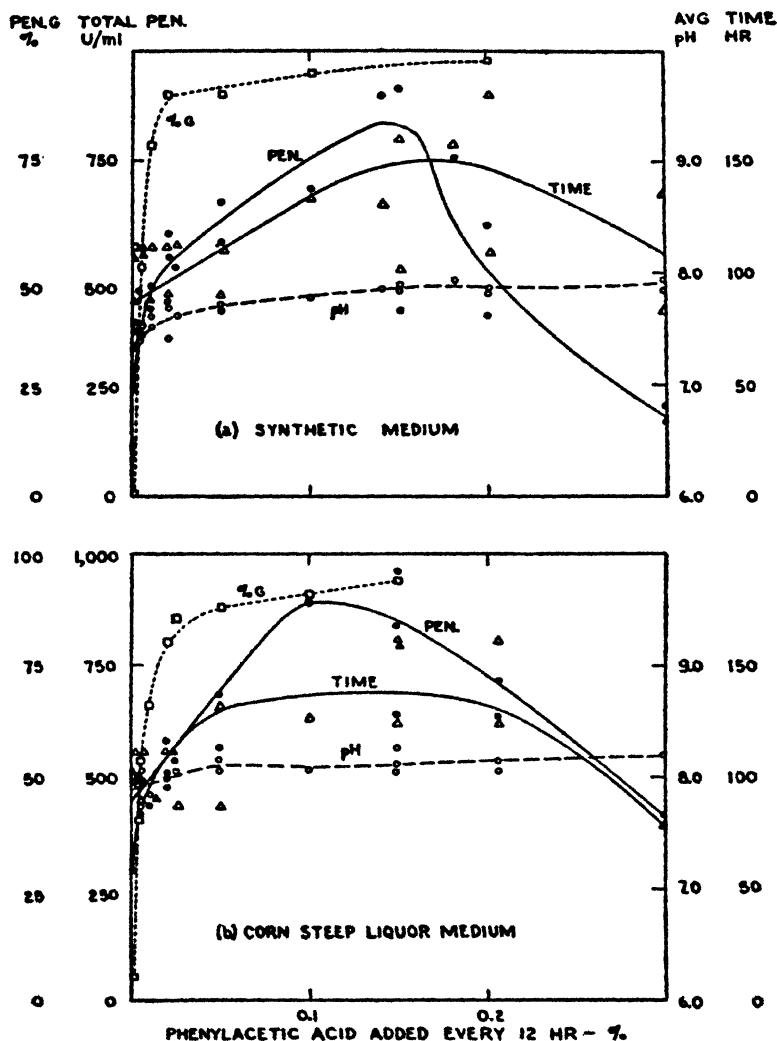
Figure 5. Effect of addition rate of phenylacetic acid on penicillin yield, proportion of G, pH, and time of fermentation.   Phenylacetic acid was added every 12 hours beginning at the fifteenth hour and a total of 9 to 10 additions were made.   ●———● Total penicillin, u per ml.   □ - - - - □ Penicillin G, per cent.   O— — —O pH.   △———△ Time to reach peak yield, hours.

no increase in penicillin yield was observed when these adapted inocula were used
in fermentations in the presence of phenylacetic acid.

Effect of pH on penicillin yield and proportion of penicillin G.   To find the opti-
mum pH for the penicillin production under optimum concentration levels of

phenylacetic acid, different pH levels were obtained by changing the lactate, acetate, and phosphate content of the medium. The ammonia content was held constant by the addition of ammonium sulfate. As shown in table 2, maximum penicillin yields were obtained at a pH range of 7.6 to 7.9. The time required to obtain the peak yield also followed the same pattern as the penicillin yield. Whether the variation in lactate, acetate, phosphate, and sulfate content had any

TABLE 2

Effect of pH during the fermentation on penicillin yield

PHENYLACETIC ACID[*]	CONC. OF VARIABLES			AVG pH	MAXIMUM PEN. YIELD	TIME OF FERMENTATION
	Amm. lactate	Amm. acetate	Amm. sulfate[†]			
per cent	*g/L*	*g/L*	*g/L*		*u/ml*	*hr*
A. 0.10	0.0	1.0	5.0	4.20	2	12
	0.0	2.5	4.15	5.90	60	39
	0.0	2.0	4.6	6.72	242	135
	5.5	3.5	0.0	7.78[‡]	685[‡]	140[‡]
B. 0.15	5.5	0.0	3 0	5.60	2	12
	0.0	1.0	5.0	5.60	4	12
	0.0	2.0	4.6	5.80	4	12
	0.0	2.5	4.15	7.15	393	159
	0.0	3.0	3.75	7.15	428	122
	1.2	3.0	3.0	7.40	565	122
	2.4	3.0	2.26	7.50	480	122
	5.5	1.0	2.15	7.60	209	95
	3.6	3.0	1.53	7.60	563	122
	5.5	2.0	1.29	7.65	508	122
	5.5	3.5	0.0	7.75	417	111
	5.5	3.5	0.0	7.80	609	143
	5.5	3.5	0.0	7.80	891	183
	5.5	3.0	0.43	7.80	769	183
	5.5	3.5	0.0	7.83	915	159
	5.5	3.0	0.43	7.90	685	143
	6.0	3.5	0.0	8.05	236	95

[*] Added every 12 hours (9 additions).

[†] Ammonium sulfate was added to make up for the deficiency of ammonia when ammonium lactate or acetate content was reduced.

[‡] Average of 12 runs of 3 flasks each.

effect other than that attributable to pH is not known. Pratt (1945) and Pratt and Hok (1946) have emphasized the importance of keeping a proper balance of KH_2PO_4, $MgSO_4$, and $NaNO_3$ for maximum yields, but it is unfortunate that their data do not include determinations of pH during the fermentation.

The effect of pH during the fermentation on the availability of the precursor and hence of the proportion of penicillin G is shown in figure 6. A comparison of two pH levels at a low precursor level (figure 6a) shows that, at a higher pH, both the yield and percentage of penicillin G are increased, indicating the increasing availability of the precursor. In figure 6b, comparison of two fermentations at

a higher precursor level shows a yield difference but no difference in the percentage of penicillin G.   The data are consistent with the assumption that, although
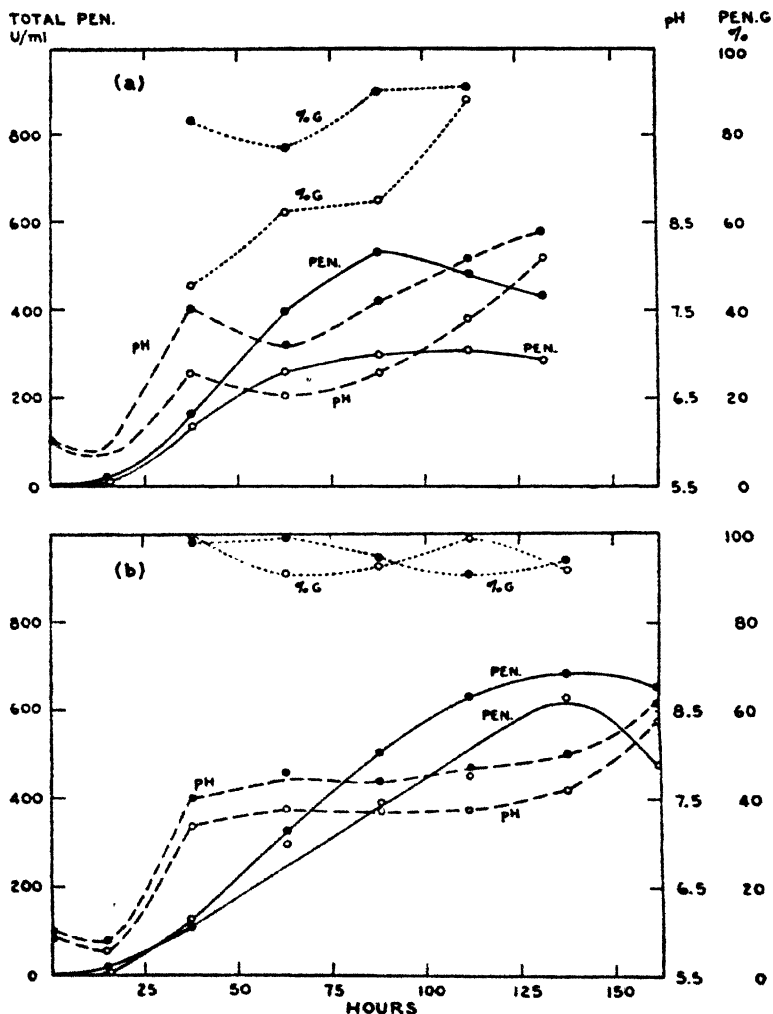
Figure 6. Effect of pH on penicillin yield and precursor efficiency of phenylacetic acid. *a*. Precursor added in 10 equal portions, 0.02 per cent each every 12 hours, beginning at the fifteenth hour.   *b*. Precursor added in 8 equal portions, 0.10 per cent each every 12 hours, beginning at the fifteenth hour.   ●————● The medium contained (in grams per liter): lactic acid 4.5, ammonium acetate 3.5, KH₂PO₄ 3.0.   O————O The medium contained (in grams per liter): lactic acid 3.0, ammonium acetate 3.0, KH₂PO₄ 6.0.

phenylacetic acid is a more efficient precursor at high pH values, in the presence of sufficient precursor the penicillin formed is predominantly G even at lower pH values.

Use of other penicillin G precursors.   It has been stated that the maximum

yields of essentially pure penicillin G in synthetic medium can be obtained only by adding 0.10 to 0.15 per cent phenylacetic acid at approximately 12-hour intervals. This amount of precursor is roughly 50 to 75 times more than that recovered as penicillin G. A small percentage of this could be found in the broth after the fermentation, but most of the phenylacetic acid (71 to 94 per cent) was lost (table 3). The use of high concentrations is, therefore, uneconomical. Another disadvantage is that the peak yields are somewhat delayed in the presence of high concentrations. Furthermore, frequent additions are inconvenient.

To overcome these difficulties, several precursors of penicillin G mentioned under "Experimental Methods" were tested to see if some of them could be used in comparatively smaller amounts without adversely affecting the penicillin yield and proportion of penicillin G. The results obtained with the use of derivatives of phenylacetic acid in two concentration levels are shown in tables 4 and

TABLE 3

Loss of phenylacetic acid during the fermentation

P.A.* ADDED	TOTAL P.A. ADDED	PEN. G PRODUCED	P.A. THEO-RETICALLY REQUIRED	FREE† P.A. PRESENT	P.A. META-BOLIZED
	g/L	u/ml	g/L	g/L	per cent
0.05% at 24 hr‡.........................	0.5	209	0.0512	0.0250	84.76
0.10% at 24 hr‡.........................	1.0	226	0.0553	0.1135	83.10
0.14% at 24 hr.........................	1.4	399	0.0978	0.0900	86.59
0.70% at 24 hr.........................	7.0	490	0.1200	0.3000	94.00
0.02% at 12-hr intervals (9 additions)...	1.8	480	0.1175	0.4000	71.25
0.10% at 12-hr intervals (9 additions)...	9.0	641	0.1517	1.3500	83.25
0.10% at 12-hr intervals (9 additions)...	9.0	672	0.1647	1.4000	82.26

* P.A. denotes phenylacetic acid.
† Thanks are due to Dr. K. Higuchi for the determinations of free phenylacetic acid.
‡ These fermentations were run by Dr. Higuchi on synthetic medium.

5. The precursors are divided into 3 groups (esters, amides, and others) and are arranged within the group in decreasing order of efficiency. The values for the control without precursor and for an equivalent amount of phenylacetic acid are included for reference. It will be seen that, although the penicillin yields at the lower concentration of phenylacetic acid and three of the precursors (phenylacetamide, phenylacetylglycine, and phenylacetyl-DL-alanine) are nearly equal, the proportion of penicillin G with the derivatives is low. Furthermore, concentrations of phenylacetamide equivalent to 0.28 per cent or more proved to be toxic.

Phenylacetylmethylamine, phenylacetyl-β-phenylethylamine, phenylacetonitrile, β-phenylethyl alcohol, and phenylacetaldehyde were found to be toxic when they were added at the time of inoculation or at 24 hours. Smith and Bide (1948) had earlier reported the last three compounds to be effective as precursors, but the conditions of their experiments are not available at the time of writing.

Though no stimulation in yield was apparent when DL-phenylalanine was added

to the medium, its function as a penicillin G precursor is evident from the fact that penicillin G comprised about 24 per cent of the total yield.   It was found to be toxic at the higher level used.

TABLE 4

Effect of derivatives of phenylacetic acid on penicillin yield, proportion of G, pH, and time of fermentation

(Precursor concentration equivalent to 0.14 per cent phenylacetic acid; added at 24 hours)

PRECURSOR	PEAK YIELD	AVERAGE pH	TIME FOR PEAK YIELD	PEN. G*
	u/ml		*hr*	*per cent*
1. Control........................	242	7.30	87	0.0
2. Phenylacetic acid..............	521	7.75	111	
	423	7.57	111	
	490	7.43	87	81.5
Esters				
3. Octadecanol phenylacetate..........	315	6.9	87	
	383	7.32	111	27.05
4. Ethyl phenylacetate..............	35	8.13	39	
5. Isopropyl phenylacetate .	35	8.13	39	
6. Butyl phenylacetate	35	8.13	39	
7. Isobutyl phenylacetate ..	35	8.13	39	
8. Glycol diphenylacetate..	22	8.0	39	
	16	8.3	39	
Amides				
9. Phenylacetamide...	590	7.50	111	
	504	7.53	87	71.0
10. Phenylacetyl-DL-alanine..	483	7.43	87	57.0
11. Phenylacetylglycine..	461	7.47	87	56.1
12. Phenylacetylmethylamine..........	79	7.90	39	
13. Phenylacetyl-β-phenylethylamine...	29	8.00	39	
Others				
14. β-Phenylethylamine†	428	7.58	111	51.0
15. DL-Phenylalanine..	220	7.74	90	24.1
16. β-Phenylethylalcohol...	52	7.97	39	
17. Phenylacetaldehyde..	18	8.20	39	
18. Phenylacetonitrile........	16	8.30	39	

* At the time of maximum yield.

† This was added at 15 hours.

Of the esters tested, those of ethyl, isopropyl, butyl, and isobutyl alcohols and of ethylene glycol were completely inhibitory.   When these esters were added at 24 hours, the pH rose higher than 8.0; but the pH remained at 6.0 when ester addition was made at the time of inoculation.   Some stimulation was observed with the octadecanol ester, but the yield and proportion of penicillin G were considerably lower when compared to those obtained with equivalent amounts of phenylacetic acid.

In a number of experiments with these precursors, penicillin G determinations were made at intervals during the fermentation.   In figure 7 are shown the data obtained when precursors were added at 24 hours in concentrations equivalent to 0.14 per cent phenylacetic acid, and table 6 summarizes experiments in which the concentration of precursors added was equivalent to 0.70 per cent phenyl-acetic acid.   Comparable data obtained with β-phenylethylamine and phenyl-acetic acid as precursors are given in figures 3 and 4, respectively.   The results

TABLE 5

Effect of derivatives of phenylacetic acid on penicillin yield, proportion of G, pH, and time of fermentation

(Precursor concentration equivalent to 0.70 per cent phenylacetic acid; added at 24 hours)

PRECURSOR	PEAK YIELD	AVERAGE pH	TIME FOR PEAK YIELD	PEN. G*
	u/ml		*hr*	*per cent*
1. Control........	242	7.30	87	0.0
2. Phenylacetic acid...............	604	7.75	111	92.7
	529	7.65	111	
Ester				
3. Octadecanol phenylacetate. ... {	348	6.87	87	
	463	7.40	111	57.4
Amides				
4. Phenylacetyl-DL-alanine.......	490	7.67	111	78.6
5. Phenylacetylglycine...	418	7.65	63	74.5
6. Phenylacetamide..	14	7.90	39	
Others				
7. β-Phenylethylamine†	87	6.39	185	
8. DL-Phenylalanine...............	57	8.14	63	

* At the time of maximum yield.
† This was added at the time of inoculation.

indicated that the precursors could be placed in three distinct groups in regard to their availability or precursor efficiency:

(1) Those whose efficiency remains practically constant during the entire fer-mentation up to the time of peak yield, e.g., phenylacetic acid and phenylacetam-ide.   As phenylacetate is the active group, it appears that the phenylacetamide is hydrolyzed rapidly, thus maintaining the high percentage of penicillin G.

(2) Those whose efficiency increases with the progress in fermentation, e.g., phenylacetylglycine and phenylacetyl-DL-alanine.   This is probably due to a slow rate of hydrolysis, so that the effective concentration of the active group becomes the limiting factor.   The comparatively higher percentage of penicillin G with phenylacetyl-DL-alanine in the initial stages indicates that the rate of its hydrolysis is faster than the rate with which phenylacetylglycine is hydrolyzed.

(3) Those whose efficiency decreases with the progress in fermentation, e.g., β-phenylethylamine, DL-phenylalanine, and octadecanol phenylacetate.   This

might be due to slow destruction or degradation of the precursor, since it has been established that a high percentage of penicillin G can be obtained if β-phenylethylamine is added in small amounts at intervals.
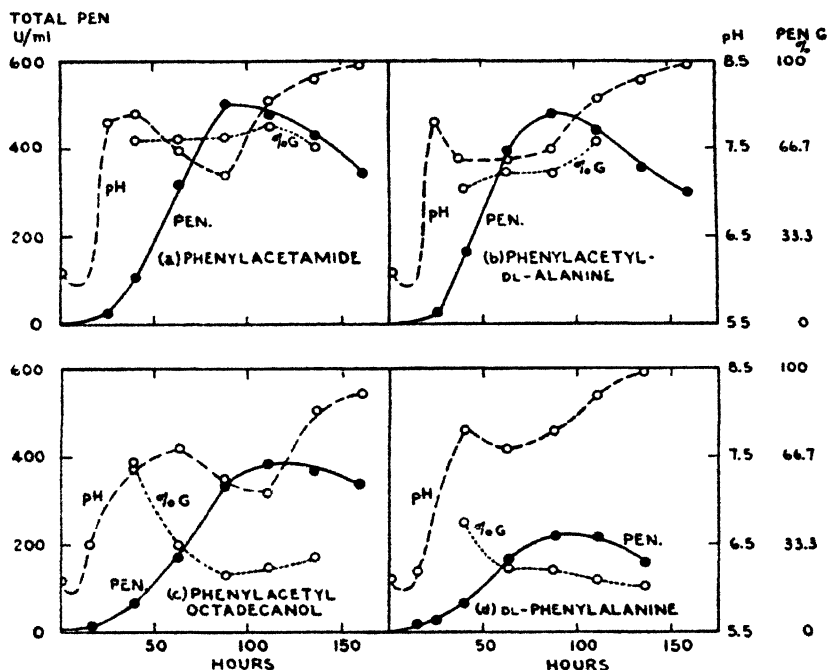
Figure 7. Comparison of efficiency of some typical precursors. Precursors added at 24 hours in concentrations equivalent to 0.14 per cent phenylacetic acid.

TABLE 6

Comparison of efficiency of some precursors

(Precursor concentration equivalent to 0.70 per cent phenylacetic acid; added at 24 hours)

	PRECURSOR								
TIME	Phenylacetyl-DL-alanine			Phenylacetyl-glycine			Phenylacetyl-octadecanol		
	Total pen.	pH	Pen. G	Total pen.	pH	Pen. G	Total pen.	pH	Pen. G
hr	u/ml		per cent	u/ml		per cent	u/ml		per cent
39	120	7.4	64.3	160	7.4	45.8	107	7.5	87.4
63	357	7.3	81.0	418	7.9	74.5	300	7.4	63.6
87	454	7.8	74.4	340	8.3	79.5	404	7.2	51.4
111	490	8.2	78.6	246	8.5		463	7.5	57.4
134	367	8.5	90.4		8.7		447	8.0	50.4

SUMMARY

Marked increases in total penicillin yield and proportion of penicillin G were obtained in submerged cultures of *Penicillium chrysogenum* Q176 in a synthetic medium by the addition of β-phenylethylamine and phenylacetic acid. No

penicillin G was detected when no precursor was added. Maximum precursor efficiency was obtained when the precursor was added in many equal portions at intervals. The optimum concentration of phenylacetic acid to be added at 12-hour intervals was 0.10 to 0.15 per cent and penicillin yields (practically pure G) averaged 685 u per ml on synthetic medium and 892 u per ml on corn steep liquor medium. From 71 to 94 per cent of the added phenylacetic acid was metabolized in ways other than the production of penicillin G. The optimum pH range during the fermentation was 7.6 to 7.9. The percentage of penicillin G was low at an acid pH.

Attempts to effectively replace phenylacetic acid with some of its derivatives have been unsuccessful. The percentage of penicillin G remained practically constant during the course of fermentation with phenylacetic acid or phenyl-acetamide as precursors. It increased during the fermentation with phenylac-etylglycine and phenylacetyl-DL-alanine but decreased with β-phenylethylam-ine, DL-phenylalanine, and octadecanol phenylacetate.

Phenylacetic acid seems to be the best precursor as far as availability, peni-cillin yield, proportion of penicillin G, and ease in handling are concerned.

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STREPTOMYCIN-DEPENDENT BACTERIA IN THE IDENTIFICATION OF STREPTOMYCIN-PRODUCING MICROORGANISMS

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There are many strains of *Streptomyces griseus*, but only a few of them produce streptomycin. The differentiation of the streptomycin-producing strains from the others is not a simple procedure and is usually time consuming (Waksman *et al.*, 1946a,b). Because of this, there has been a need for finding a rapid, reliable method of identifying the streptomycin-producing strains of *S. griseus*. Recently Waksman and his group (1947) have successfully employed an actinophage that is specific for streptomycin-producing strains of *S. griseus*. The present report describes the use of certain streptomycin-dependent bacteria in a simple and accurate procedure for the identification of streptomycin-producing strains of *S. griseus*.

While we were studying a strain of *Mycobacterium ranae* that grows only in the presence of streptomycin (Yegian and Budd, 1948), it occurred to us that this microorganism would be useful in identifying streptomycin-producing strains of *S. griseus* if it could be shown that its growth in the presence of actinomycetes and other microorganisms is limited to those producing streptomycin. Unfortunately the relatively slow rate of growth and other characteristics of *M. ranae* made it unsuitable for the purpose. Through the courtesy of Dr. Paine (1948) we acquired strains of four rapidly growing microorganisms that required the presence of streptomycin for their growth: *Staphylococcus aureus* (S7-1), *Escherichia coli* (E158), *Pseudomonas aeruginosa* (PY24), and *Proteus morgani* (PR24). Of these the streptomycin-dependent strains of *E. coli* and *P. aeruginosa* proved well suited for the rapid and accurate identification of streptomycin-producing strains of *S. griseus*. The streptomycin-dependent strains of *P. morgani* and *S. aureus* were unsuitable for our purpose chiefly because with repeated subculturing a few colonies were found that grew in the absence of streptomycin.

EXPERIMENTAL RESULTS

Cultures and media. The actinomycetes used in these experiments were a streptomycin-producing strain of *Streptomyces griseus* (3496); a mutant strain of *Streptomyces griseus* (3495) that no longer produces streptomycin but another antibiotic; *Streptomyces lavendulae* (3440-14), which produces streptothricin; *Streptomyces violaceus-ruber* (3033) which produces no antibiotic; and *Streptomyces griseus* (3478), which produces grisein. These cultures were obtained through the courtesy of Dr. Waksman. In addition, other microorganisms, obtained from the soil and food products or found as laboratory contaminants, were used; these included some forms known to produce antibiotic substances.

This selection of different microorganisms was used to determine whether the streptomycin-dependent bacteria would grow in the presence of microorganisms other than streptomycin-producing *S. griseus*.

Streptomycin-dependent strains of *P. aeruginosa* and *E. coli* were used as test organisms throughout this experiment.

Slants made from a modified form of FDA medium (Stebbins and Robinson, 1945) containing 1.5 per cent agar and 100 μg per ml of streptomycin were found best suited for maintenance of the stock cultures of the bacteria. The plates used in the experiments were made using 20 ml of the modified FDA medium in each plate. Nutrient broth containing concentrations of streptomycin in different quantities was used to obtain liquid growth of the bacteria.

Procedure and results. Tubes of liquid medium containing 100, 50, 10, 5, 2.5, and 0 μg per ml of streptomycin were inoculated with saline suspensions of the stock cultures of *E. coli* and *P. aeruginosa*. Turbid growth in 24 hours was obtained in tubes containing 100, 50, and 10 μg per ml of streptomycin. Some growth in 24 hours was obtained in tubes containing 5 μg per ml of streptomycin. No growth, even after 48 hours, was obtained in tubes containing 2.5 and 0 μg per ml of streptomycin. In order to obtain strains of these bacteria capable of growth in the presence of small quantities of streptomycin, tubes containing 2.5 μg per ml of streptomycin were inoculated with organisms growing in the presence of 5 μg per ml of streptomycin, and in 24 hours an adequate growth was obtained. Liquid medium containing only 1 μg per ml of streptomycin was then inoculated with organisms growing in 2.5 μg per ml of streptomycin, and in this manner strains were obtained that grew well in only 1 μg per ml of streptomycin. These strains were maintained and used as a source of test organisms. Attempts to grow these organisms in the absence of streptomycin failed, and we believe the strains are rather stable.

Plates of solid medium containing no streptomycin were inoculated with the different actinomycetes, a loopful of a saline suspension of a different culture being placed in the center of each plate. The plates were incubated for 48 hours, after which a growth of 1 to 2 cm in diameter was evident. A loopful of the liquid growth of one of the test bacteria was streaked on the plates, starting at the periphery of the agar and ending at the edge of the actinomycete's growth in the center. In this way several streaks of each of the test organisms could be made on a single plate (figure 1). Initially we had centrifuged the liquid cultures, removed the supernatant, and washed the bacteria several times in saline to remove all traces of streptomycin. This procedure was found to be unnecessary for the purpose of the experiment, for no growth of the streptomycin-dependent organisms ever occurred on the plates except adjacent to the streptomycin-producing microorganisms.

The plates containing the actinomycete's growth and the test bacteria were incubated at 37 C, and after 24 hours bacterial growth was noted adjacent to streptomycin-producing strains of *S. griseus*, but nowhere else. There was a gradual decrease in the profuseness of the bacterial growth as it extended from the center, and no growth was noted beyond 2 cm from the border of the actinomycete. There was never growth of the streptomycin-dependent bacteria on a

plate containing an organism other than a streptomycin-producing strain of *S. griseus*.

Figure 1 Showing growth of streptomycin-dependent *E. coli* only in the region immediately surrounding a streptomycin-producing strain of *S griseus* The bacteria were inoculated by streaking from the periphery of the plate to the edge of the actinomycete's growth.

Figure 2. Showing 3 strains of *E. coli* on a plate containing a central growth of a streptomycin-producing strain of *S. griseus*. *A*, a strain of streptomycin-dependent *E. coli* showing growth only in the portion of the streak near the actinomycete. *B*, a strain of *E. coli* resistant to streptomycin. Growth is evident all along the streak. *C*, a strain of *E. coli* susceptible to streptomycin showing lack of growth in the portion of the streak near the actinomycete.

Figure 3. Showing the spread of growth of streptomycin-dependent *P. aeruginosa* about a growth of a streptomycin-producing strain of *S. griseus*. The bacteria were inoculated at the tip of the *S. griseus* streak.

Bacteria known to be susceptible to streptomycin were streaked on plates containing streptomycin-producing strains of *S. griseus*, and they grew only in the regions separated from the actinomycete, whereas the densest growth of the streptomycin-dependent strains occurred where they were closest to the actinomycete (figure 2).

Several modifications of the foregoing procedure were tried and the results were always similar. In one modification a suspension of streptomycin-dependent bacteria was spread over the entire surface of a number of plates that contained no streptomycin. The plates were incubated and no growth was visible after 24 hours. Several different actinomycetes were inoculated on each plate so that each inoculum was well separated from the others. After an additional incubation of 48 hours the actinomycetes' growths were evident, and after still further incubation bacterial growth was visible in zones surrounding streptomycin-producing strains of *S. griseus*. Bacterial growth was never noted elsewhere on any of the plates.

An interesting phenomenon was observed when streptomycin-dependent *P. aeruginosa* was inoculated adjacent to a growth of streptomycin-producing *S. griseus:* the bacterial growth gradually spread during a period of 5 days from the site of inoculation until it completely encircled the actinomycete's growth (figure 3).

DISCUSSION

The foregoing experiments demonstrate that so-called streptomycin-dependent strains of certain rapidly growing bacteria are suitable for the rapid and accurate identification of streptomycin-producing strains of *S. griseus*. The streptomycin-dependent strains of *E. coli* and *P. aeruginosa* never grew in a medium that did not contain a source of streptomycin. The test bacteria would not multiply about growths of the microorganisms tested unless they produced streptomycin. The presence of other antibiotics produced by various organisms did not permit the growth of the streptomycin-dependent bacteria that were tested.

It was essential for the experiment to select strains of *E. coli* and *P. aeruginosa* sufficiently sensitive to grow rapidly in the presence of small amounts of streptomycin. Many more cells capable of growth in 2.5 μg per ml of streptomycin were growing in 5 μg per ml of streptomycin than were present in the original stock cultures that were growing in the presence of 100 μg per ml of streptomycin. Similarly, more bacteria capable of growth in 1 μg per ml of streptomycin were growing in 2.5 μg per ml of streptomycin than in 5 μg per ml of streptomycin. Growth in decreasing concentrations of streptomycin is probably related to the selection of naturally occurring variants.

The peculiar spontaneous spreading of the growth of the dependent *P. aeruginosa* until it covered the entire zone about the streptomycin-producing strain of *S. griseus* is of interest. The streptomycin located in the medium appears to determine the direction of spread of the bacterial growth.

We have found that the dependent organisms' requirement for streptomycin can be fulfilled when body fluids from patients being treated with streptomycin are used. When serum or urine is used in the cylinder plate method of assay and one of the dependent strains is substituted for the sensitive SM staphylococcus Merck, there is a zone of bacterial growth about the cylinders, and the diameter of the growth is directly related to the concentration of the streptomycin in the serum or urine.

The presence of occasional variants capable of growth in the absence of streptomycin has been noted in cultures of streptomycin-dependent strains. However, the frequency of these variants is low in our test strains and has not caused interference with the experiments.

SUMMARY

The use of streptomycin-dependent strains of certain bacteria for identifying streptomycin-producing strains of *Streptomyces griseus* has been described. The streptomycin-dependent strains never grew in the presence of any microbe other than streptomycin-producing strains of *S. griseus*. The technique is simple, and accurate results are obtained rapidly.

Streptomycin-dependent strains of bacteria can be readily selected to grow in very low concentrations of streptomycin.

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CARBON ASSIMILATION TESTS FOR THE CLASSIFICATION OF YEASTS

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For the past several years the senior author has been engaged in a study designed to improve techniques used in the classification of yeasts. Reports have already been issued regarding certain phases of this work, namely, studies on fermentation tests (1943) and nitrogen assimilation tests (1946). The present paper, dealing with carbon assimilation tests, represents the third in this series. These papers dealing with techniques will be followed by a second series covering the classification of the various groups of yeasts.

In the above-mentioned report on the nitrogen assimilation tests (1946), it was shown that by the addition of an adequate supply of pure vitamins to the test medium some yeasts would assimilate nitrogenous compounds that they were previously reported incapable of assimilating. The results indicated that the use of ammonium sulfate, urea, asparagine, and peptone as test substances should be discontinued, since they are always assimilated. The present paper is similar in nature but deals with the assimilation of carbon sources. A more adequate basal medium and a greater number of carbon compounds have been employed than were used by earlier investigators, with the result that this type of biochemical test is proving to be the best that has been developed for the classification of the yeasts.

Up to the present time the carbon sources used in assimilation tests in the major attempts at yeast classification have been limited to glucose, fructose, mannose, galactose, maltose, sucrose, lactose, and ethyl alcohol. A number of workers have attempted to use pentoses, polysaccharides, organic acids, and polyhydric alcohols, but the results have been quite variable among strains of the same species. There has as yet been no comprehensive testing of large numbers of strains of many species, followed by an analysis of the data to determine which compounds give consistent, taxonomically valuable results in each group of related species. This is the objective of the work reported in this paper.

The carbon assimilation tests have been used for many decades. Perhaps the first study was made by Beijerinck (1889), and the Dutch workers are still using his technique, though somewhat modified, as given on page 74 of Diddens and Lodder's monograph (1942). In this technique a synthetic agar medium that has been inoculated with a heavy suspension of cells is solidified in a petri dish. After the surface is dry, small amounts of various common sugars are placed on the agar. Growth develops in the areas where the assimilable compounds were placed. Those yeasts that show poor growth are retested on the same medium

to which some yeast extract has been added.   The results are usually read after 1 or 2 days of incubation.

Okunuki (1931) used 1-ml quantities of a synthetic agar medium in test tubes. He employed 16 different carbon sources, including pentoses, alcohols, and some of the more complex polysaccharides in addition to the common sugars used by the Dutch workers.   This investigator recorded the relative amounts of growth after 5 days of incubation.   Okunuki also tested the ability of his yeasts to utilize five organic acids.   A base medium containing peptone and salts was used, and the amount of acid that disappeared during the 35-day incubation period was determined by titration.

Liquid cultures have been employed by a number of investigators, for they are less subject to contamination than plate cultures and can therefore be incubated longer.   Zikes (1906) used liquid yeast extract media containing 5 and 10 per cent of the carbon sources.   The cultures were incubated 4 weeks, then observed for growth.   Redaelli and Ciferri (1929) used a neutral Raulin solution containing 1 per cent of the carbohydrate, alcohol, or acid.   They recommended determination of the pH of the medium and the volume of the cells after centrifugation of 10-day-old cultures.   Bedford (1942) employed a synthetic liquid medium that permitted only very poor growth with some species.   Nickerson (1943) determined the ability of yeasts to utilize carbon sources by the Warburg manometric technique.

The literature cited above reveals marked variations in media, incubation periods, and methods of determining whether the carbon compounds have been assimilated.   For other modifications the reader is referred to the works of Langeron and Guerra (1938, p. 164), Zimmermann (1938), and Mackinnon (1946, p. 20).

EXPERIMENTAL METHODS

Preliminary work over a period of years demonstrated the following requirements for obtaining satisfactory results in the carbon assimilation tests.

(1) Pure vitamins added to a synthetic medium will allow growth of many species that otherwise would fail to grow.   The pure vitamins themselves yield no detectable growth in the absence of assimilable carbon.   Yeast extract or other vitamin carriers, such as have been used by some investigators to supply the requirements of the yeasts, support sufficient growth in the absence of any test substance to give difficulty in reading the tests.

(2) The incubation period should be at least 18 days, and preferably 24, for alcohols, pentoses, and organic acids are assimilated more slowly than the majority of common hexoses and disaccharides.

(3) The yeast under test should be conditioned to grow in the synthetic medium with glucose before it is subjected to similar synthetic media containing carbon sources that may be difficult for the yeast to attack.

The basal medium now used is nearly the same as that described in the study of the nitrogen assimilation tests (1946), and only information regarding additional procedure is given in the present paper.   The two phosphate salts have been replaced by the single salt, monopotassium phosphate, in concentration of

1 gram per liter. This gives the medium a pH of approximately 5.0. The trace elements manganese, in the form of manganese sulfate quadrihydrate, and molybdenum, in the form of sodium molybdate dihydrate, are added to the extent of 0.10 part per million of manganese and 0.01 part per million of molybdenum. Five-tenths per cent ammonium sulfate serves as the nitrogen source. Each carbon compound under test is used in a concentration having an amount of carbon equal to the carbon in a 0.5 per cent glucose solution, except for a few compounds of low solubility that were used in saturated condition. The amount of raffinose was doubled since utilization might involve only a part of the molecule.

Tenfold amounts of the ammonium sulfate, potassium dihydrogen phosphate, and sodium chloride are added to 90 ml of distilled water, as described in the article referred to above (1946). The carbon compound is likewise added in 10-fold concentration, and if it is an acid or acid salt the pH is adjusted to 5.2 by the addition of sodium hydroxide pellets to avoid dilution. The solution is then sterilized by passage through a Seitz filter. Ten ml of 100-fold vitamin solution are added. The resulting solution is dispensed aseptically in 0.5-ml quantities into 16-mm tubes containing 4.5 ml of a solution containing trace elements, magnesium sulfate, and calcium chloride. The medium is then ready for inoculation. Soluble starch and inulin are dissolved by warming in the solution of trace elements, pipetted into test tubes, and then sterilized in the autoclave for not more than 20 minutes.

One company has prepared in dehydrated form the medium just described. The desired amount is dissolved in distilled water, the carbon source is added, and the medium is sterilized by passage through a Seitz filter. Then it is pipetted into test tubes containing 4.5 ml of sterile distilled water, and the medium is ready for use. This company has produced two similar media, one for use in nitrogen assimilation tests and one for use in determining morphological characteristics. In our experience these dehydrated media have proved satisfactory, and if they are placed on the market we believe they should find wide acceptance for studies in yeast classification.

The yeast to be tested is grown on a slant of yeast extract, malt extract agar of the following composition: 3 g of powdered yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of glucose, and 20 g of agar in 1 liter of distilled water. The pH lies between 5.0 and 6.0, depending on the batch of ingredients used. All incubation is at 25 C, for some species grow erratically at higher temperatures. After 48 hours, transfer is made to 10 ml of the complete synthetic medium containing as carbohydrate only 0.1 per cent of glucose. One ml of the liquid medium is placed on the slant, the growth is emulsified by means of the pipette, and 0.2 ml to 0.4 ml of the suspension, depending on the abundance of the growth on the slant, are transferred to the tube of synthetic medium. This culture is also incubated for 48 hours, permitting the cells to become adapted to growth in a synthetic medium, and presumably at the same time reducing their carbohydrate reserves. The culture is then diluted with synthetic medium that contains no carbohydrate. The inoculum is diluted to a light transmission of

approximately 26 per cent by using the Aminco type F photometer and its no. 42 filter. With this photometer the dilution is made directly in the 20-by-175-mm tubes used for growing the inoculum. Twenty-six per cent transmission corresponds to approximately 23 million cells per ml of *Saccharomyces cerevisiae*, NRRL strain Y-567, or 33 million cells per ml of *Torulopsis utilis*, Y-900, as determined by hemacytometer counts. One-tenth ml of the inoculum is used to inoculate each tube. It imparts no cloudiness.

The tests are incubated for 24 days, this long incubation period being necessary to the development of certain characteristically latent, adaptive enzyme systems. The tests are read at 7 and 24 days. The tubes are shaken, then placed against a white 3-by-5-inch filing card on which are drawn with India ink lines approximately one-half millimeter wide. If the growth in the tube completely obliterates the line, it is recorded as 3+; if the line appears through the growth as an indistinct band, it is recorded as 2+; if the line can be distinguished as such, but the edges of the line are indistinct, the growth is recorded as 1+, and if the edges can be distinctly seen it is recorded as negative. This gives a record of latent reactions in the data book; for publication purposes a 3+ or 2+ reaction at 24 days is recorded simply as +, and a 1+ reaction is recorded as W(eak). Little taxonomic importance is attached to a W reaction because it may be due to impurities in the carbon source.

Preliminary tests were made on some 70 compounds using 100 strains of yeasts representing 22 genera. Then one or more strains were studied of each species of yeast in the NRRL collection, using those compounds listed in groups 7, 8, and 9 under "Results." Finally, a study of the reactions of many strains of all genera was started and is still in progress.

RESULTS

The carbon sources tested may be roughly classified in the following groups. In these groups the small capital letters D and L refer to configuration.

(1) Compounds that were not assimilated by any of the test yeasts: cyclohexanol, cyclohexane carboxylic acid, tricarballylic acid, sodium dihydroxy tartrate, maleic acid, kojic acid, diglycolic acid, pinacol, ethylene glycol, pentaerythritol, methyl alcohol, acetone, ethyl citrate, nicotinic acid, choline chloride, and alpha-methyl-D-mannoside.

(2) Compounds used by too few yeasts to warrant their use in routine diagnosis: itaconic acid, malonic acid, levulinic acid, adipic acid, pimelic acid, L-ascorbic acid, mucic acid, glutaric acid, D-gulono-α-lactone, calcium D-glucoheptonate, glycogen, disodium phenyl phosphate, calcium lactobionate, and D-glucosamine hydrochloride.

(3) Compounds used by approximately half of the genera of yeasts, but which do not appear to have sufficient value for use in routine diagnosis: L-malic acid, fumaric acid, and L-glutamic acid.

(4) Compounds that gave too many latent and weak reactions: DL-aspartic acid.

(5) Compounds that gave variable reactions because of toxicity: acetic acid.

(6) Compounds that gave indefinite reactions because they are used incompletely by some yeasts but completely by others: amygdalin, D-glucoascorbic acid, and 5-desoxy-L-sorbose.

(7) Compounds of most promise for taxonomic work: D-xylose, D-arabinose, L-arabinose, D-ribose, L-rhamnose, L-sorbose, cellobiose, melibiose, trehalose, inulin, soluble starch, DL-erythritol, adonitol, dulcitol, D-mannitol, D-sorbitol, calcium 2-keto-D-gluconate, potassium 5-keto-D-gluconate, potassium D-gluconate, succinic acid, DL-lactic acid, and citric acid.

(8) Compounds of less promise for taxonomic work: ethyl alcohol, glycerol, pyruvic acid, ethyl acetoacetate, DL-inositol, and sodium potassium saccharate.

(9) Supplementary compounds: melezitose and alpha-methyl-D-glucoside for additional information on alpha-glucosidase activity, and salicin for additional information on beta-glucosidase activity.

The method of evaluating the compounds in the last three groups is to run them with a large number of strains in each genus of yeasts. Then those compounds that give the fewest variable reactions and permit the greatest degree of differentiation between species will be selected for routine classification work. Glucose, galactose, maltose, sucrose, lactose, and raffinose are also included.

Let us consider the application of this method to the genus *Hansenula*. It is a particularly good one because speciation is made especially clear by the variety of spore shapes and fermentation reactions. These characters may be used as a check on speciation as indicated by the assimilation reactions. In the forthcoming paper on the genus *Hansenula*, nine species and one variety will be described. *Hansenula anomala* is the most commonly occurring, and over 70 strains have been studied. All strains except the variety *schneggii* assimilated glucose, maltose, sucrose, trehalose, cellobiose, raffinose, melezitose, soluble starch, ethyl alcohol, glycerol, erythritol, mannitol, sorbitol, alpha-methylglucoside, salicin, and pyruvic, lactic, succinic, and citric acids. Most strains assimilated galactose, xylose, ribose, adonitol, and potassium gluconate. L-Arabinose was usually not assimilated. No strains assimilated sorbose, lactose, melibiose, inulin, D-arabinose, rhamnose, dulcitol, calcium 2-ketogluconate, potassium 5-ketogluconate, potassium sodium saccharate, ethyl acetoacetate, and inositol. Similarly, all other species of the genus were examined to determine which compounds gave consistent reactions with all strains and which were variable. Then a table was prepared in which were entered all species and the compounds that gave invariable or nearly invariable reactions. From this table, five compounds were selected that gave good separation between species on reactions that are invariable insofar as the compounds for the specific separations are concerned.

These compounds are galactose, sorbose, raffinose, rhamnose, and alpha-methylglucoside. Rhamnose is of particular interest because it provides for the first time a biochemical means of differentiating between *Hansenula saturnus* and *Hansenula suaveolens*, and between *Hansenula anomala* and *Hansenula ciferrii*. It is of interest to note that eight species do not have unique fermentation reactions insofar as the genus is concerned, yet all but two species (*H. anomala* and *H. subpelliculosa*), which are separated by a combination of differences in spore

shapes and fermentation reactions, can be separated on differences in assimilation reactions alone.

Studies on all other genera will be made subsequently in a manner similar to the one on *Hansenula*, after which a selection will be made of the carbon sources best adapted for all routine classification of yeasts. Studies in progress at the present time have already revealed great differences in the number of carbon compounds assimilated by different genera. *Debaromyces* species utilize nearly all of the compounds. *Pichia, Mycoderma*, and all of the apiculate yeasts utilize only a few. The apiculates are of especial interest because every strain utilizes cellobiose, even though it may use only two to six other compounds, depending on the species to which it belongs.

TABLE 1

Spore shapes and fermentation and assimilation reactions of the species of the genus Hansenula

SPECIES	SPORES	FERMENTATION REACTIONS					ASSIMILATION REACTIONS				
		G	Ga	M	S	R1/3	Ga	So	R	Rh	Me*
H. beckii.............	Hat	—	—	—	—	—	—	—	—	+	+
H. minuta.... ...	Hat	+	—	—	—	—	—	—	—	—	—
H. californica.....	Saturn, thin rings	+	—	—	—	—	—	+	—	+	+
H. mrakii.	Saturn, thick rings	+	—	—	—	—	—	—	—	+	—
H. silvicola.... .	Hat	+	+	—	—	—	+	+	—	+	+
H. suaveolens....	Spheroidal	+	—	—	+	+	—	—	+	—	—
H. saturnus........	Saturn, thick rings	+	—	—	+	+	—	—	+	+	—
H. subpelliculosa.....	Hat	+	—	V	+	+	V	—	+	—	+
H. anomala	Hat	+	V	V	+	+	V	—	+	—	+
H. anomala var. schneggii. ..	Hat	+	—	+	+	—	+	—	—	—	+
H. ciferrii.......	Hat	+	+	V	+	+	+	—	+	+	+

* G, glucose; Ga, galactose; M, maltose; S, sucrose; R, raffinose; So, sorbose; Rh, rhamnose; Me, alpha-methylglucoside; V, variable; +, gas where it refers to fermentation, growth where it refers to assimilation.

Two examples can be cited from the genus *Candida* to illustrate how the assimilation tests reveal the inclusion of certain strains in the wrong species. *Candida guilliermondii* var. *nitratophila* was the name given by Diddens and Lodder (1942) to a strain that they believed to belong in this species even though it differed from other strains by assimilating nitrate. The proposed variety, however, differs so greatly that the species cannot encompass this strain. In addition to the difference in nitrate utilization, all strains yet studied of *C. guilliermondii* assimilate the following compounds that the variety *nitratophila* cannot assimilate: galactose, sorbose, melibiose, L-arabinose, ribose, rhamnose, dulcitol, and calcium 2-ketogluconate. Similarly, *Candida krusoides* has been placed in synonymy with the species *Candida krusei* by Diddens and Lodder. Both ferment only glucose. However, *C. krusoides* assimilates galactose, sorbose, malt-

ose, sucrose, trehalose, melezitose, L-arabinose, mannitol, sorbitol, and calcium 2-ketogluconate, none of which were assimilated by the 12 strains of *C. krusei* thus far tested.

Numerous examples could be cited to demonstrate how the assimilation tests indicate the desirability of reducing to synonymy species now recognized.

It is of interest to indicate the role of vitamins and trace elements in the carbon assimilation tests. Bedford (1942) in his valuable study of the genus *Hansenula* added no vitamins to his assimilation test media, with the result that *Hansenula subpelliculosa* and *Zygohansenula californica* grew poorly and failed to reduce nitrate in it. All strains tested of these two yeasts reduced nitrate actively and

ZYGOHANSENULA CALIFORNICA
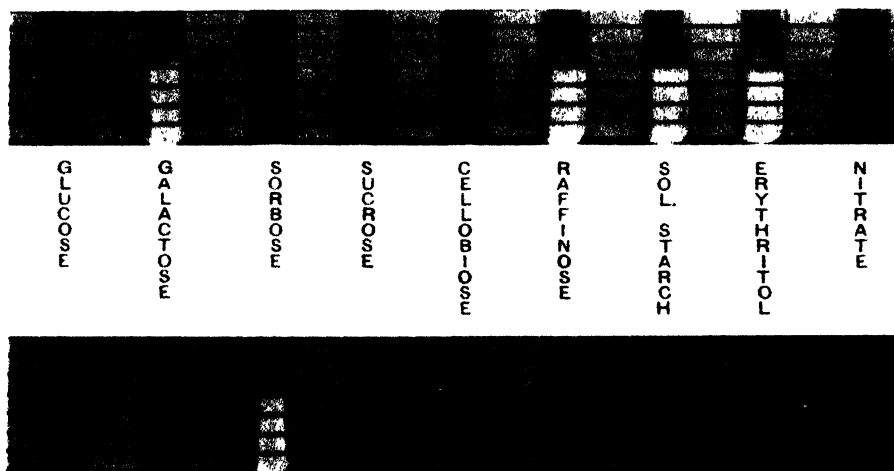
HANSENULA SUBPELLICULOSA

Figure 1. Two species of yeasts, previously reported incapable of good growth in a synthetic medium, grow well if pure vitamins are added. Differences are demonstrated in the ability to assimilate the various carbon sources.

grew satisfactorily in the medium used in the present study, as illustrated in figure 1.

Endomycopsis fibuliger, Endomycopsis selenospora, Endomycopsis javanensis, Zygosaccharomyces rugosus (one strain), *Zygosaccharomyces versicolor,* and *Saccharomyces dairensis* (one strain) are the only yeasts thus far studied that will not grow on the synthetic medium used in this study. At the time this paper was being written, we found that the first four of the above-mentioned species will grow in the synthetic medium if DL-methionine, L-cystine, or glutathione is added; the last two will grow if L-tryptophan is added. Methionine and tryptophan are now being added to the synthetic medium in a concentration of 10 milligrams of each amino acid per liter of medium.

The addition of trace elements appears to have little influence on growth. A number of fastidious yeasts have been tested in assimilation medium modified to

contain no added trace elements as well as combinations of trace elements covering a wide range of concentrations. There was no significant difference in the amount of growth obtained.

It is therefore concluded that sufficient trace elements are present as impurities in the other ingredients. Nevertheless the addition of trace elements will be continued for protection against the future when manufacturers may have eliminated some essential trace element from their chemicals.

DISCUSSION

The expanded use of assimilation tests in the classification of yeasts has made them the most valuable type of biochemical test thus far developed. The results are easily and swiftly read without the use of reagents or photometer. There are no complications such as are found in fermentation tests in which a reversal of reaction may be encountered, or in the nitrate reduction reaction in which the nitrite may have a transitory existence. Once the assimilation tests become positive they remain positive. The assimilation tests are valuable in both nonfermentative and fermentative genera, for results depend upon the ability of the individual to attack a compound and not on the recognition of one of the many metabolites resulting from the attack.

During the course of this work tests have been run on molds not far above the yeasts in morphological development. In general, these have given strong, characteristic reactions. Bacteria likewise might be studied advantageously by this technique, using appropriate buffer, vitamins, trace elements, and, when necessary, the minimum requirement of amino acids and purine or pyrimidine compounds. The authors have obtained interesting results on a few strains of algae without modification of the medium.

ACKNOWLEDGMENT

The authors acknowledge with pleasure the technical assistance of Mr. Richard Gill and the photographic work done by Roland Haines, photographer for the Northern Regional Research Laboratory.

CONCLUSIONS

The number of compounds employed in the assimilation tests has been increased.

The additional compounds are proving to be of much value in the classification of yeasts.

The expanded assimilation tests are suggested as having possible usefulness in the classification of bacteria, molds, and algae.

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NOTES

A SINGLE SOLUTION INDICATOR FOR ANAEROBIOSIS[1]

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In the preparation of insecticides using methylene blue as a coloring agent, it was observed that some preparations, when heated, brought about reduction of the dye, which became reoxidized immediately on being shaken in air. Investigation indicated that the reduction of the methylene blue was caused by the combined action of glucose and sodium monohydrogen orthoarsenate ($Na_2HAsO_4 \cdot 7H_2O$). Preparations containing various concentrations of the two reagents and methylene blue were tested. The most satisfactory contained:

Glucose.....	4 g
$Na_2HAsO_4 \cdot 7H_2O$..	2 g
Methylene blue (85%)...	0.003 g
Distilled water..	100 ml

A small amount of the solution was put into a test tube or small flask and heated gently to bring about reduction of the dye just before it was placed in the anaerobic jar. The small amount of methylene blue which became oxidized at the top of the indicator solution during manipulations and before anaerobiosis occurred would be reduced during incubation if the oxygen was removed completely from the jar.

The original pH of the solution was 8.45. After ten subsequent reductions by heating and reoxidations on standing in contact with air, the pH fell stepwise to 7.48, at which point the indicator still possessed the original activity. This series of redox changes was carried out in approximately 8 hours. A common three-solution indicator system consisting of a combination of sodium hydroxide, glucose, and methylene blue solutions, and similar to that of Fildes (*System of Bacteriology*, vol. IX, Med. Research Council, Brit., 1931), was prepared and treated in the same manner. The pH of the solution was 10.0 before heating, and after 7 subsequent reductions and oxidations the pH fell to 6.85. From the third reduction each succeeding decoloration of the dye became more difficult, until, after the seventh reduction, it was no longer possible to reduce the methylene blue.

Glucose arsenate indicator and alkaline glucose indicator were repeatedly reduced and allowed to stand open to the air for 24 to 72 hours before being reduced again. In one series, the glucose arsenate indicator was decolorized 13 times during 24 days, and at the end of this period was still easily reduced and reoxidized. The alkaline glucose completely lost its activity in 3 days, but it was rather difficult to reduce the methylene blue even after 24 hours. A second series gave similar results, except that the glucose arsenate indicator that was reduced

17 times in 24 days showed some fading of the methylene blue on the last reoxidation.

The stability of the glucose arsenate indicator system over longer periods of time was studied, and indications are that the solution will remain stable and allow good decoloration and reoxidation to the original color up to at least 11 months. A single solution of the alkaline glucose methylene blue loses its activity in 2 weeks or less if it is not kept from contact with air.

From the foregoing, it appears that a single solution of glucose arsenate methylene blue indicator appears to possess more desirable properties for indicating anaerobiosis than the three-solution alkaline glucose methylene blue system.

MUTATION TO TRYPTOPHAN INDEPENDENCE IN EBERTHELLA TYPHOSA

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The training of bacteria to grow in the absence of a previously required metabolite has been considered as due either to slow, continuous adaptation or to the occurrence of spontaneous mutations. We have reinvestigated the training of *Eberthella typhosa* to dispense with tryptophan in order to test the applicability of the latter point of view.

E. typhosa, strain O 901, Vi-negative (obtained through the courtesy of Dr. J. Craigie) gives full growth in semisynthetic medium with 4 micrograms per milliliter of DL-tryptophan, and a trace of visible turbidity with 0.2 micrograms per ml. The organisms can be trained to dispense with tryptophan by transfer in media with diminishing concentrations of the amino acid. As the result of plating washed cells of the dependent strain grown in a complete medium, a few colonies appear, containing cells capable of growth in minimal medium without tryptophan. The numbers of these independent variants in parallel cultures, each started from one cell, showed fluctuations in the frequency of the variants (table 1), such as would be expected from the analysis of spontaneous mutation given by Luria and Delbrück (Genetics, 28, 491). The mutation rate was calculated as suggested by these authors, and values between 1.4 and 7.5×10^{-8} mutations per cell per generation were obtained. Media with concentrations of tryptophan of the order of 0.1 micrograms per ml allow partial growth of the dependent cells. If one or more mutants arise, they proceed to grow, reaching a concentration higher than that of the normal cells. The replacement of dependent by mutant cells in media with concentrations of the order of 0.1 microgram per ml of tryptophan is likely to represent the critical step in "training" experiments. The mutants retained the tryptophan independence through 30 daily transfers plus routine transfers in complete medium for over a year.

This work was presented in partial fulfillment of the requirements for the degree of Master of Arts at Indiana University. The author is grateful to Dr. S. E. Luria for advice, and to the Indiana Federation of Women's Clubs for a fellowship.

TABLE 1

Determination of the frequency of mutant cells in normal cultures

Seven flasks with 50 ml of nutrient broth were each inoculated with a well-isolated colony of *Eberthella typhosa* O 901, and incubated with shaking for 36 hours. The cells were harvested and washed twice by centrifugation (30 minutes at 2,500 rpm). The cell deposits were resuspended in 10 ml of sterile 0.85 per cent NaCl solution, and used to inoculate plates and tubes of minimal medium. The titer of each suspension was determined by colony count.

	CULTURE NO.						
	1	2	3	4	5	6	7
Inoculum, cells per plate	3.2×10^8	1.8×10^8	2.6×10^3	1.4×10^8	2.6×10^8	1.3×10^8	1.1×10^8
Colonies of the mutant in individual plates	106	2	0	0	0	1	0
	130	0	1	0	0	0	1
	135	0	0	1	0	1	0
	117	1	0	0	0	5	0
		1	0	0	0	0	0
		0	0	0	0	0	0
		0	2	0	2	0	0
		0	2	0	0	1	0
		0	3	0	0	1	0
		0	0	0	0	4	1
Average	122	0.47	0.8	0.1	0.2	1.3	0.2
Ratio: Mutant cells/normal cells	3.8×10^{-7}	2.2×10^{-9}	3×10^{-9}	7×10^{-10}	7.7×10^{-10}	1×10^{-8}	1.8×10^{-9}
Smallest inoculum that gave growth in liquid medium, cells per tube	1.2×10^6	3.6×10^5	2.6×10^6	2.8×10^5	5.2×10^5	2.6×10^5	2.2×10^5
Calculated no. of mutants in the inoculum that gave growth in liquid medium	0.45*	0.79*	0.78*	0.19*	0.4*	2.6*	0.39*

* The average of the figures followed by an asterisk is 0.8.

THE RELATIONSHIP BETWEEN PRODIGIOSIN PRODUCTION AND CATALASE ACTIVITY

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Prodigiosin, the pigment of *Serratia marcescens*, is agreed to be a tripyrrole methene. Waring and Werkman (Arch. Biochem., **1**, 428) have shown that iron

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deficiency in the medium inhibits pigment formation. They have also shown (Arch. Biochem., 4, 75) that catalase activity in *Aerobacter indologenes* falls with decreasing iron content. There exist variant strains of *Serratia marcescens* that do not produce pigment, and it appeared possible that a comparison of colored and uncolored variants could be used to demonstrate a direct relationship between pigment production and catalase activity.

Catalase activities of our normal stock pigmented strain and a white variant that had appeared spontaneously and bred true through continuous transfers were compared on pour plates by means of Oxford cups containing hydrogen peroxide. Table 1 shows the differences noted in the diameter of zones of inhibition.

Heavy saline suspensions of the two strains were studied spectrophotometrically in the Coleman photoelectric spectrometer. Two absorption curves of the pigmented strain were prepared. For the first curve, the white strain was placed in the reference cell to obtain a comparative absorption curve for the pigmented strain against the white variant. To obtain the second curve, a

TABLE 1

CONCENTRATION OF HYDROGEN PEROXIDE	DIAMETER OF ZONE OF INHIBITION AFTER 24 HOURS AT ROOM TEMPERATURE	
	White variant	Normal form
per cent	*cm*	*cm*
3	4.5	3.5
0.3	1.9	1.3
0.03	0.75	0.75

suspension of the normal strain that had been kept unpigmented by growth at 37 C was used in the reference cell. It was assumed that this curve would reveal differences of absorption that were due solely to the pigment. Both curves followed the general shape of that for prodigiosin (Ehrismann and Noethling: Biochem. Z., 284, 381). The normal red against white curve had a number of sharp peaks, which did not in most instances appear on the other curve. In contrast, the normal red against unpigmented red curve showed plateaus at these points, indicating identical absorption characteristics. A series of peaks at 430, 505, 540 to 550, and 630 mμ was interpreted as the catalase absorption spectrum.

The two experiments outlined above show that the normally pigmented strain of *Serratia marcescens* has a considerably higher catalase activity than the unpigmented variant, and that there appears to be a correlation between ability to produce pigment and catalase activity.

Assuming the porphin nucleus to be synthesized *in vivo* by the same steps that have been used in the laboratory synthesis, the following is proposed as a hypothetical explanation of the origin of prodigiosin:

$$\text{Tripyrryl methanes} \longrightarrow \text{Prodigiosin}$$
$$\downarrow\uparrow$$
$$\text{Substrate} \longrightarrow \text{Dipyrryl methenes} \longrightarrow \text{Porphin} \longrightarrow \text{Catalase}$$
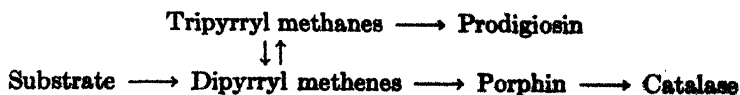
The production of tripyrryl methanes during the laboratory synthesis of dipyrryl methenes has been demonstrated by Corwin and Andrews (J. Am. Chem. Soc., **58**, 1086). It is suggested that when catalase production exceeds the demand for it, the equilibrium may shift, favoring an increase in concentration of the intermediate di- and tripyrryl stages. This suggestion is supported by the following experimental observations: (1) The pigment is produced at room temperature, and only by old cells. (2) The absorption curve of the normal red against the white variant showed a sharp peak at 450 mμ, corresponding to the absorption maximum given for alkyl dipyrryl methenes (Granick and Glider: Advances in Enzymol., **7**, 360). (3) There is a lack of pigment production when growth of the normal strain occurs at 37 C. The recognized instability of catalase at this temperature could result in reducing the amount of active enzyme to such an extent that the envisaged side reaction production of prodigiosin would not occur.

A BIOCHEMICAL AND SEROLOGICAL STUDY OF A GROUP OF IDENTICAL UNIDENTIFIABLE GRAM-NEGATIVE BACILLI FROM HUMAN SOURCES

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In the course of a study of paracolon bacilli a number of cultures were submitted to this laboratory as possible paracolon organisms. Included in this series were 15 identical cultures of gram-negative bacilli that could not be identified. Representative strains were sent to Dr. Kenneth Wheeler and by him to Dr. C. A. Stuart. Dr. Wheeler reported the organisms to be an unidentifiable *Bacterium* species and Dr. Stuart confirmed this report, stating that similar gram-negative bacilli had been encountered in his laboratory, where they were known as "5W." Because of the not infrequent occurrence of these unidentifiable organisms in clinical material from human sources and because the apparent homogeneity of the group suggests the presence of a hitherto undescribed species, the biochemical and serological study to be reported here was undertaken.

The source of the cultures included in the group to be described is given in table I. Figures on their incidence are not available, but their common occurrence in clinical material is evidenced by the fact that in one month 11 strains, 8 of them from urine cultures, were submitted to us for identification by other laboratories of the Johns Hopkins Hospital.

Morphology. Morphologically these organisms are short, fat, coccoid, gram-negative bacilli, showing marked bipolar staining. In newly isolated strains the morphology is regular, and true bacillary forms are seldom seen. In gram-stained preparations the organisms strongly resemble *Neisseria* and morphological differentiation would be difficult if not impossible; in fact, one strain in our series, isolated from the cervix, was received from another laboratory to which it had been submitted as *Neisseria gonorrhoeae*, and several other strains were regarded as possible *Neisseria* species on first isolation. Hanging drop preparations, however, usually demonstrate the true bacillary form of the organisms.

Morphologically stock cultures vary from newly isolated strains in showing definite bacillary forms as well as the coccoid form described above. Rough forms of the organisms are quite pleomorphic, showing coccoid, bacillary, and irregular, curved, filamentous forms, whereas small-colony variants are made up entirely of long, irregular, twisted filaments.

These organisms naturally occur in the "M," encapsulated phase. The capsules are quite small but are easily demonstrated in India-ink preparations. Growth on media known to encourage capsule formation, such as agar plus ascitic fluid with or without milk and glucose, failed to increase the size of the capsule or alter the gross colony morphology. No motility has been demonstrated in repeated studies on each strain.

Growth characteristics. The gram-negative bacilli of this group are strict aerobes, growing well aerobically and at reduced oxygen tension, but failing completely to grow anaerobically. In broth culture they grow heavily with an even turbidity, slight smooth sediment, and a delicate pellicle. The pellicle increases on prolonged incubation, and in 24 to 48 hours a heavy ring is formed. No pigment is produced on solid or liquid media at room or incubator temperatures.

On blood or pancreatic digest agar (Brown, 1948) streak plates, well-isolated colonies are large (2 to 3 mm), white, opaque, raised, convex, smooth, and glistening, with an entire edge. In blood agar pour plates deep colonies are small and biconvex, and there is no hemolysis or discoloration of the blood around either

TABLE 1

Source of cultures

STRAIN NUMBER	SOURCE	CLINICAL IMPRESSION
8	Urine	Urinary tract infection
25	Urine	Urinary tract infection
77	Urine	History not available
80	Left pleural cavity—autopsy	Aspiration pneumonia, atelectasis
81	Urine	Urinary tract infection
83	Pleural and pericardial fluids—autopsy	Postoperative empyema
85	Urine	Urinary tract infection
86	Urine	Urinary tract infection
87	Cervix	Gonorrhea?
90	Urine	History not available
93	Urine	Urinary tract infection
94	Urine	History not available
95	Urine	Urinary tract infection
97	Lung—autopsy	Capillary bronchitis, terminal bronchopneumonia
113	Heart blood—autopsy	Capillary bronchitis

deep or surface colonies. On desoxycholate agar the organisms present a distinctive appearance. In general, the colonies are similar to those described above, but they are unique in that small colonies are colorless or yellowish, whereas larger, well-isolated colonies usually show a definite pink color or pink center in spite of the fact that 1 per cent lactose broth is not fermented. Prolonged incubation of blood agar or desoxycholate agar plates frequently results in the production of small daughter colonies. Transfers from these secondary colonies yield small-colony variants, which will be described later.

Because of the striking morphological resemblance of these organisms to *Neisseria*, they were also studied on chocolate agar plates. Well-isolated colonies on this medium were large and opaque, resembling those on other media. The oxydase test was completely negative on all strains.

Biochemical study. A study of the cultural characteristics of these gram-

negative bacilli has shown them to be biochemically identical.   In Durham tubes containing 1 per cent carbohydrates in extract broth, only glucose, galactose, and arabinose were found to be fermented.   Fermentation consistently occurs in a characteristic fashion, a slight acid reaction appearing first at the surface of the broth in the outer tube and gradually extending to the bottom of the medium.   No growth or fermentation occurs in the inner tube.   Glucose is fermented in 24 hours, galactose and arabinose in 2 days.   Lactose, sucrose, xylose, mannitol, salicin, maltose, inositol, trehalose, sorbitol, adonitol, raffinose, rhamnose, and dulcitol were not fermented after 1 month's incubation.

When the organisms of this group were first studied, we were investigating the use of 10 per cent lactose agar slants for the identification of paracolon bacilli as recommended by Chilton and Fulton (1946), and these strains were therefore studied on this medium.   Although 1 per cent lactose broth or agar slants were consistently negative after prolonged incubation, 10 per cent lactose agar slants were found to be regularly fermented in 24 hours.   This apparent discrepancy was later thoroughly investigated and found to be characteristic of these organisms.   Ten per cent lactose agar slants show rapid fermentation regardless of the method used for the preparation of the medium, whether the lactose is autoclaved in the complete medium, autoclaved in aqueous solution and added aseptically to the agar, or whether a filtered lactose solution is used.   The latter two methods were investigated to eliminate possible breakdown of the lactose during sterilization, and the medium containing 10 per cent lactose so prepared was readily fermented in 24 hours.   Varying the pH of the medium from 7.0 to 7.7 also failed to influence the results.   Ten per cent lactose broth tubes were fermented more slowly, requiring from 3 to 5 days' incubation.   However, if freshly inoculated 10 per cent lactose broth cultures were poured into sterile petri dishes to increase the available oxygen, fermentation occurred in 24 hours as on the 10 per cent lactose agar slants.

A group of control cultures, consisting of a number of strains each of *Escherichia, Aerobacter, Eberthella, Shigella, Salmonella, Proteus, Pseudomonas, Pasteurella*, and *Alcaligenes*, were also studied on 10 per cent lactose agar slants.   *Escherichia* and *Aerobacter* fermented the medium as would be expected, whereas all strains of *Eberthella, Shigella, Salmonella, Pseudomonas, Pasteurella*, and *Alcaligenes* were consistently negative.   *Proteus vulgaris, Proteus mirabilis*, and *Proteus rettgeri* were also negative, but all strains of *Proteus morganii* gave an acid reaction after 48 hours' incubation on all 10 per cent lactose agar slants regardless of the method of preparation.   Therefore, with the exception of a similar but delayed reaction by *Proteus morganii*, fermentation of 10 per cent lactose agar slants correlated with failure to ferment 1 per cent lactose broth or agar appears to be a unique characteristic of these unidentifiable gram-negative bacilli.

As for the other biochemical characteristics of these organisms, all strains gave the following results: indole negative, methyl red positive, Voges-Proskauer negative, citrate positive, nitrates negative, malonate positive, and hydrogen sulfide negative.   Urease is produced on Christensen's urea agar (1946), a $++$

reaction developing in 24 hours. Gelatin is liquefied slowly, requiring from 6 weeks' to 2 months' incubation. In milk plus bromcresol purple, acid is produced in 3 to 4 days and coagulation in 1 week. There is complete inhibition of growth on Difco SS agar slants inoculated with undiluted cultures. The failure to reduce nitrates is an outstanding characteristic of these organisms and was checked carefully. Cultures were incubated for periods varying from 24 hours to 5 days, and the negative tests were checked with zinc dust (Frobisher, 1944), which in every case showed unreduced nitrates to be present.

It might also be mentioned here that determination of penicillin sensitivity by the agar streak plate, penicillin disk technique of Bondi, Spaulding, Smith, and Dietz (1947) showed these organisms to be highly resistant to penicillin, since there was no inhibition of growth around the penicillin-saturated disk.

Variants. The various strains of these gram-negative bacilli have been carried in stock for a year since their original isolation. During this time variation was noted in several strains. An "S" form has been found that resembles the original "M" form morphologically with the exception of loss of capsules, and varies culturally only in delayed fermentation of glucose and galactose. An "R" form also occurs that shows morphological pleomorphism, no capsules, typical granular growth in broth with loss of pellicle and ring formation, and marked delay in the fermentation of carbohydrates, from 3 to 6 days being required to ferment glucose, arabinose, and galactose. The "R" form also utilizes malonate more slowly than typical strains and fails to produce urease or to liquefy gelatin.

As previously mentioned, prolonged incubation of agar plates results in the production of secondary colonies, isolations from which proved to be small-colony variants. These variants produce very small, streptococcuslike colonies on blood agar and desoxycholate agar plates, and in broth grow poorly with a slight turbidity and sediment and no ring or pellicle. Morphologically they are unencapsulated, long, irregular, extremely pleomorphic filaments with few bacillary forms present. These small-colony variants fail to ferment glucose, galactose, and arabinose, and 10 per cent lactose agar slants, and also vary from the typical organisms in being malonate-, urease-, and gelatin-negative. Only agglutination in high titer with specific antiserum prepared against a typical strain confirms the relation of these small-colony variants to typical organisms of this group.

Serological study. Antiserum was prepared by rabbit inoculation against a typical encapsulated strain of these gram-negative bacilli. Quellung tests set up in the usual manner showed an immediate, marked swelling of the capsule with all strains in the "M," encapsulated phase. The unencapsulated, smooth, rough, and small-colony variants naturally failed to give a positive quellung test. The quellung reaction with encapsulated strains, in which form the organisms naturally occur, offers a rapid method of identification, since quellung tests on a group of control cultures consisting of representative strains of *Escherichia, Aerobacter, Eberthella, Salmonella, Shigella, Pseudomonas,* and *Pasteurella* were all negative.

Cross-agglutination tests resulted in agglutination to the titer of the serum with

all "M" strains and 80 per cent agglutination of the small-colony variant. The smooth and rough strains failed completely to agglutinate. Agglutinin-absorption experiments were then performed. The antiserum was absorbed with each of the "M" and small-colony variant strains, and the resulting absorbed serum was tested for agglutinins against these same cultures. It was found that each of the typical encapsulated strains completely absorbed the agglutinins against all other such cultures. The small-colony form, however, showed little agglutinin absorption, the titer of the absorbed serum being only slightly reduced with the exception of complete absorption of agglutinins against the homologous absorbing strain.

The serological study clearly demonstrates that in the encapsulated phase these organisms are serologically homogeneous. The smooth, rough, and small-colony forms, however, vary serologically from the "M" strains as might be expected. Agglutinin tests with the same series of control cultures listed above showed absolutely no cross agglutination even in low dilutions with but one exception. Strains of *Pasteurella multocida* were agglutinated to titer with the antiserum prepared against the organisms under discussion. This apparent serological relationship was investigated and it was found that (1) antisera prepared against strains of *Pasteurella multocida*, which agglutinated *Pasteurella* strains in high titer, failed completely to agglutinate these unidentifiable gram-negative bacilli; (2) absorbed antisera, which were prepared in the agglutinin-absorption experiments described above and which were found to contain no agglutinins against these organisms, agglutinated *Pasteurella multocida* strains to the same titer as the unabsorbed serum; and (3) strains of *Pasteurella multocida* failed completely to absorb the agglutinins against these organisms. Thus it was clearly demonstrated that there is no serological relationship between these gram-negative bacilli and *Pasteurella multocida* and that the agglutination observed in the cross-agglutination experiments was probably due to naturally occurring antibodies against *Pasteurella* in the rabbit serum. Since rabbits are liable to *Pasteurella* infections, the occurrence of such antibodies does not seem improbable.

As for the agglutination of these unidentifiable organisms by antisera prepared against recognized species of gram-negative bacilli, none has been observed to date. This fact was confirmed by Wheeler (1947), who reported that "the serology is unrelated to anything we have on hand." He specifically reported no agglutination with antiserum against the mannitol-negative and mannitol-positive groups of *Shigella* or against the *Shigella dispar* and *Shigella alkalescens* groups, which is of some interest since these organisms resemble *Shigella* in some biochemical characteristics.

Pathogenicity for animals. The pathogenicity of these unidentifiable gram-negative bacilli was tested in mice and guinea pigs. Mice succumb to intraperitoneal injection of 0.01 milliliter of a 24-hour broth culture in less than 24 hours, whereas guinea pigs are killed by a similar injection of 0.25 ml. The organisms were regularly recovered from the heart blood, pleural fluid, and peritoneum of the dead animals and were identified culturally and serologically.

TABLE 2

Differentiation of Bacterium anitratum and culturally similar gram-negative bacilli

ORGANISM	COLONIES ON DESOXYCHOLATE AGAR PLATE	MOTILITY	LACTOSE	GLUCOSE	SUCROSE	XYLOSE	MANNITOL	INDOLE	METHYL RED	VOGES-PROSKAUER	CITRATE	MALONATE	NITRATES	UREASE*	10% LACTOSE AGAR SLANTS	MILK PLUS B.C.P.	SS AGAR	ANAEROBIC GROWTH	QUELLUNG AND AGGLUTINATION TEST WITH B. ANITRATUM ANTISERUM
Bacterium anitratum	Usually pale pink; few strains colorless or yellowish	-	-	A	-	-	-	-	+	-	+	alk	-	+	+	A, coag. 5-10 days	-	-	+
Pseudomonas	Colorless colonies becoming brown in 48-72 hours	+	-	- or sl. A	-	-	-	-	-	-	+	alk	++†	+	-	alk., pep.	+	-	-
Pasteurella	No growth	-	-	A	A	A	A	+	+	-	-	-	+	-	-	sl. A	-	+	-‡
Shigella	Colorless colonies	-	- or late A	A	-	-	A/-	+/-	+	-	-	-	+	-	-	sl. A	+	+	-
Bacterium parapertussis	Colorless colonies	-	-	-	-	-	-	-	-	-	+	-/A	-	+	-	sl. alk.	-	-	-

A = acid, alk. = alkaline; sl. = slight; +/- = may be positive or negative.

* Urease production determined on Christensen's urea agar.

† Nitrite test is negative, but addition of zinc dust shows no unreduced nitrates present; therefore, both nitrates and nitrites are reduced.

‡ Positive agglutination test demonstrated to be due to naturally occurring agglutinins against Pasteurella in the rabbit serum.

DISCUSSION

Biochemically these gram-negative bacilli appear to differ from any recognized genera and species described in *Bergey's Manual of Determinative Bacteriology*, sixth edition. A possible relationship to *Pseudomonas* has been suggested, but the organism here described produces no pigment; there are well-defined morphological and biochemical differences and agglutination tests show no serological relationship. In table 2 is shown the differentiation of these organisms from *Pseudomonas*, and also from *Pasteurella, Shigella*, and *Bacterium parapertussis*, to which they may bear a superficial resemblance.

As the taxonomic position of these gram-negative bacilli is not clear, it seems advisable at this time to place them in the genus *Bacterium*, which is reserved for organisms not yet assigned to any of the recognized genera. Since it is their failure to reduce nitrates that differentiates them from the great majority of gram-negative bacilli, particularly the *Enterobacteriaceae* to which they appear to be most similar, it seems appropriate to select a species name that will express this characteristic, for example, *"anitratum."* Therefore, the name *Bacterium anitratum* n. sp. is proposed as a name for this group of biochemically and serologically homogeneous gram-negative bacilli.

ACKNOWLEDGMENTS

We are indebted to Dr. Kenneth Wheeler and to Dr. C. A. Stuart for studying representative strains of these organisms, and to Dr. Gerald J. Schipper, who kindly gave us the strains of *Pasteurella multocida* and the *Pasteurella* antisera used in this study.

SUMMARY

The morphological, cultural, and biochemical characteristics of a group of unidentifiable gram-negative bacilli from human sources have been described.

Cross agglutination and agglutinin-absorption tests have shown these organisms, when in the "M," encapsulated phase, to be serologically homogeneous and to bear no serological relationship to gram-negative bacilli of the *Enterobacteriaceae* and other recognized genera.

Bacterium anitratum n. sp. has been suggested as a fitting name for these organisms.

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FURTHER STUDIES ON THE GRAM STAIN

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In 1886 Christian Gram devised a staining technique for differentiating bacterial species. The nature of the action of the stain upon bacteria has been of considerable interest, and many theories have been proposed concerning the mechanism of the staining reaction. One theory is that of Jobling and Petersen (1914), who are of the opinion that gram-positive bacteria are richer in unsaturated fatty acids than are other bacteria. They argue that the addition of iodine at the double bonds forms an alcohol-insoluble combination with the unsaturated fatty acids in the "ectoplasm" and thus renders the cell wall impermeable to alcohol. This impermeability prevents the removal of the dye that has been absorbed. Another theory is that of Benians (1912). He believes that the effect of the iodine is to dissociate the dye from its adsorption compound with the tissues to form a large molecular body. The iodine and the dye combine. This dye-iodine complex diffuses out of gram-negative bacteria upon alcohol application but does not pass out of gram-positive bacteria.

The importance of proteins in bacterial chemistry, as related to the gram stain, was the basis of the theory of Stearn and Stearn (1928). They demonstrated that the proteins of gram-positive bacteria exhibit an isoelectric point further on the acid side of neutrality than do the proteins of gram-negative bacteria. In either case the isoelectric point is on the acid side; thus both types of organisms are stained with basic dyes. However, the gram-positive group will hold the stain more securely since the iodine shifts the pH further to the acid side and therefore increases the strength of the original staining. Thus, after treatment with iodine, the alcohol does not wash out the violet stain as readily from gram-positive bacteria.

The "modern" conception of the gram-staining mechanism was proposed by Churchman (1929). He concluded that the protoplasm of all bacteria is gram-negative and that the gram-positive bacteria are covered by an envelope of gram-positive material. This hypothesis led to more recent work. Henry and Stacy (1943) demonstrated that by using bile salts they could strip off the gram-positive outer coat from a number of bacterial species. By this treatment they were able to separate the gram-negative skeleton, which retained the shape of the organism. The gram-positive character could be restored by replating the extract back on the gram-negative skeleton. Although extract from the gram-positive cells contained carbohydrates and proteins, the most abundant constituent was magnesium ribonucleate. Henry and Stacy (1943) were also able to restore to the gram-positive state organisms rendered gram-negative as a result of bile salt treatment, by putting them into a neutral solution of the magnesium salt of pure ribonucleic acid. It is noteworthy that organisms that are normally gram-negative are not affected by this treatment and also that desoxyribonucleic

acid cannot replace ribonucleic acid in replating gram positiveness. This experiment of Henry and Stacy suggests that the gram-positive character resides in a ribonucleate complex and that the gram-negative bacteria do not contain this material.

Bartholomew and Umbreit (1944) confirmed Henry and Stacy's results and extended them further. They converted gram-positive bacteria to gram-negative forms by digestion with the enzyme ribonuclease. They were also able to replace the bacterial ribonucleate with magnesium ribonucleate prepared from yeast and thus replated the gram positiveness.

This communication reports the plating of gram positiveness upon normal gram-negative *Escherichia coli* through the action of desoxyribonucleic acid.

MATERIALS AND METHODS

The strains of *E. coli* were isolated from culture plates obtained from stool specimens of patients. The bacteria were grown in nutrient broth at a pH of 8.0.

The desoxyribonucleic acid was prepared by a modification of the method of Mirsky and Pollister (1942). The preparation when dried was light brown in color and in solution gave the most viscous solution of any preparation made in this laboratory to date, suggesting a high degree of polymerization. Solutions of desoxyribonucleic acid were prepared in sterile saline or distilled water and contained 6 mg of the acid per milliliter (of solution). All incubation was done at 37 C. Centrifugation of the bacteria was conducted at room temperature at 2,000 rpm for 30 minutes. The method of staining employed was that of Hucker and Conn (1947). All preparations were stained upon the same slide to ensure equality of conditions. The saline used in this work contained 0.85 grams of NaCl per 100 ml of distilled water.

Two 100-ml flasks of nutrient broth were each inoculated with a loopful of *E. coli* and incubated for 18 hours at 37 C. The bacteria from each portion were then centrifuged and washed 3 times each, one portion with saline, the other portion with distilled water. The portion which was washed with distilled water was suspended in 5 ml of distilled water. The portion washed with saline was suspended in 5 ml of saline. Four tubes were set up: two tubes (no. 1 and no. 2) were employed for the distilled water bacterial suspension; the remaining two tubes (no. 3 and no. 4) were used for the saline bacterial suspension. The suspensions were then treated in the following manner: To tube no. 1, 1 ml of bacteria in distilled water and 1 ml of distilled water were added. This tube served as a distilled water control. To tube no. 2, 1 ml of bacteria in distilled water plus 1 ml of desoxyribonucleic acid were added. To tube no. 3, a 1-ml portion of bacteria in saline and 1 ml of saline were added. This tube served as a saline control. Tube no. 4 contained 1 ml of bacteria in saline and 1 ml of desoxyribonucleic acid solution.

RESULTS

All control tubes contained only gram-negative bacteria. However, the organisms in the tubes containing the desoxyribonucleic acid were gram-positive

with only occasional gram-negative cells. After these gram-positive cells were washed, a further change in the staining characteristics was seen. The bacteria previously suspended in distilled water when washed 5 times with distilled water were then found to be gram-negative. However, if they were washed with saline the positiveness persisted. The same observations were noted in the bacteria originally suspended in saline; when washed with water they became gram-negative, but if washed with saline they remained gram-positive. These results are summarized in table 1.

This experiment indicates that gram positiveness can be imposed upon normal gram-negative *Escherichia coli* by the presence in the system of a highly viscous desoxyribonucleic acid. It is of importance to note that the changing of the gram-staining characteristics of *E. coli* is produced only by certain preparations of desoxyribonucleic acid, since it was shown in the course of our investigation that samples of desoxyribonucleic acid giving a less viscous solution have failed to produce the gram-staining changes.

TABLE 1

Changes in the gram stain of E. coli incubated with desoxyribonucleic acid

	TUBE 1	TUBE 2		TUBE 3	TUBE 4	
	Dist. H₂O + Bact.	Dist. H₂O + Bact. + DRNA		Saline + Bact.	Saline + Bact. + DRNA	
Gram stain..........	−	+		−	+	
		AFTER REPEATED WASHING			AFTER REPEATED WASHING	
		With H₂O	With saline		With H₂O	With saline
Gram stain..........	−	−	+	−	−	+

Desoxyribonucleic acid is soluble in distilled water but only slightly soluble in 0.85 per cent saline. This suggests a possible mechanism by which the positiveness produced upon the *E. coli* is retained after washing with saline. Since the distilled water dissolves the added desoxyribonucleic acid, it apparently removes the acid from the bacteria and thus destroys the positiveness. However, since the saline cannot dissolve the added desoxyribonucleic acid from the bacteria, the positiveness remains. Thus, it seems that the desoxyribonucleic acid combines with the *E. coli*. This combination is soluble in distilled water and insoluble in saline.

SUMMARY

Gram positiveness can be imposed upon normal gram-negative *Escherichia coli* by the addition of a highly viscous desoxyribonucleic acid.

The gram positiveness cannot be washed away with physiological saline but can be removed with distilled water.

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PROPHYLACTIC IMMUNIZATION AGAINST STREPTOCOCCUS SANGUIS

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Streptococcus sanguis (the *Streptococcus* s.b.e. of Loewe), originally identified by White and his coworkers, has, with few exceptions, been isolated only from the blood stream of patients with subacute bacterial endocarditis (White, 1944; White and Niven, 1946; Niven and White, 1946; Loewe *et al.*, 1946; Niven *et al.*, 1946; Washburn *et al.*, 1946). This causative organism has a type specificity as established by the following biological and serologic criteria: (1) it produces greening of blood agar in 24 hours; (2) it hydrolyzes arginine in 48 hours; (3) it ferments inulin within 1 week; (4) it does not ferment raffinose within 1 week; (5) it fails to produce slime on 5 per cent sucrose gelatin agar in 24 hours; and (6) it displays a type-specific precipitin reaction in immune serum within 10 to 20 minutes.

Loewe, Plummer, Niven, and Sherman (1946) and, later, Loewe and Alture-Werber (1946) reported on the prognostic significance of *Streptococcus sanguis* in subacute bacterial endocarditis and stressed the serious implications of infection with this organism. It was responsible for most of the treatment failures in our series of more than 200 endocarditis patients who received antibiotic therapy at the Jewish Hospital of Brooklyn. It was pointed out in these communications that the organism was apparently refractory to the therapy despite the fact that in the test tube it had the same range of sensitivity to penicillin or streptomycin as had other members of the *Streptococcus viridans* group. Even in the successfully treated patients massive amounts of penicillin or streptomycin, or both, were required uninterruptedly over a span of 5 to 8 weeks in order to terminate the infection.

Hehre and Neill (1946) described a dextran-producing streptococcus isolated from patients with subacute bacterial endocarditis. From their description it is highly probable that the organism is identical with *Streptococcus sanguis*. Schneierson (1948) recently confirmed the occurrence and type specificity of *Streptococcus sanguis* in a study of viridans strains isolated from patients with subacute bacterial endocarditis.

The present report concerns observations on an unselected group of 56 out of a total of 152 recovered endocarditis patients who, within the past 7 months, have been inoculated with a polyvalent vaccine consisting of 5 strains of *Streptococcus sanguis* isolated from the blood stream of patients with active bacterial endocarditis. These organisms used for preparation of the vaccine, although quite sensitive to penicillin *in vitro*, proved to be characteristically refractory *in vivo*.

This exploratory study was motivated by a desire to protect our recovered patients from the recurrences that are to be expected in this disease. Where recurrence is inevitable, it was hoped by this prophylactic program at least to avoid infection with the clinically resistant *Streptococcus sanguis.*

The 56 patients in this series were postrecovery 2 months to 5 years. The infecting organisms in this series have been tabulated as indicated in table 1.

Rabbits successfully immunized with *Streptococcus sanguis* were given three 7-day courses with 7-day rest periods. For patients a different schedule had to be devised, the first 3 injections being given intradermally, 5 million organisms per dose. All succeeding injections were given subcutaneously and the dosage was increased to 15, 25, 50, 100, 200, and 400 million organisms. Injections were given twice weekly for 4 weeks, weekly for 1 month, and, finally, every other week indefinitely; the patients are still being vaccinated. Following an increase of dosage some patients developed slight reactions, such as whealing at the site

TABLE 1

Causative organisms in 56 recovered cases of subacute bacterial endocarditis

Streptococcus sanguis	21
" *bovis*	5
" *mitis*	5
" *faecalis*	1
" not further identified	10
Staphylococcus aureus	1
Pneumococcus	1
Veillonella gazogenes	1
Spirochaeta vincenti	1
Erysipelothrix	1
Undetermined etiology	9
Total	56

of injection and swelling of the arm, which subsided in 2 to 3 days. In two patients who had reactions of longer duration, injections were withheld until the reactions disappeared and a more conservative program was then pursued.

From time to time sera were checked and titrated for agglutinin and precipitin formation. Agglutinin experiments were performed with an antigen prepared as for immunization. The bacterial cells were mixed with an equal volume of the various serum dilutions and incubated overnight, refrigerated for 2 hours, and then read. For precipitin experiments the Lancefield (1933) technique was followed. Sera diluted at least 1:10 were employed for all tests.

RESULTS

Subjects bled 4 days after the first injection were still negative (table 2). From this table one can note that with lapse of time there is a progressive rise in agglutination titers. Thirteen weeks after the start of immunization one-third of the subjects had titers of 1:20; 2 weeks later two-thirds of them had positive

titers in the 1:40 and 1:80 dilutions. At 21 weeks the titers were 1:40, 1:80, or 1:160, and remained at this level for some time; one patient at 23 weeks gave a reading of 1:320. No agglutinins were observed for a long time in 3 subjects who omitted a number of injections, but their titers started to pick up as soon as they resumed inoculations. Two subjects progressed very slowly despite a satisfactory dosage schedule; they were agglutinin-negative even after 3 and 4 months' treatment, but soon thereafter their titers rose to 1:10 and 1:20.

The same sera were tested for precipitin formation with Lancefield extracts of *Streptococcus sanguis*, the capillary method being used. Precipitins were formed more rapidly than agglutinins, and more so in humans than in rabbits. They appeared in much lower titers than the agglutinins (table 3) and seldom exceeded titers of 1:10, 1:20, 1:30, or 1:40, few reaching titers of 1:60. Seven-

TABLE 2

Results of agglutination experiments in sera of 56 subjects immunized with a polyvalent vaccine of Streptococcus sanguis

NO. OF SUBJECTS TESTED	WEEKS AFTER 1ST INJECTION	SUBJECTS WITH NEG. RESPONSE		SUBJECTS WITH POSITIVE RESPONSE IN SERUM DILUTION												
				1:10		1:20		1:40		1:80		1:160		1:320	
		no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
8	½	7	87							1	13				
50	2	27	54	8	16	9	18	4	8	1	2	1	2		
3	5					2				1					
2	9					1								1	
6	13	1	17	1	17	2	32			1	17	1	17		
20	15	1	5			3	15	7	35	7	35	2	10		
16	17	3	19	3	19	6	38	2	12	1	6	1	6		
19	19			3	16	3	16	10	52	2	11	1	5		
15	21					1	6	7	47	3	20	4	27		
25	23	1	4			1	4	6	24	13	52	3	12	1	4
20	25					2	10	7	35	8	40	3	15		
14	27					1	7	6	43	3	22	4	28		

teen weeks after the onset of immunization all sera gave typical precipitin reactions with the carbohydrate extracts of *Streptococcus sanguis*.

Table 4 presents a comparative study of agglutinin and precipitin formation in 15 patients. It is apparent that the titers of agglutinins and precipitins rose according to the duration of treatment and frequency of injections. The sera of J. H. and I. S. remained negative for a long time; these subjects interrupted their vaccine treatment but as soon as they reported regularly their titers rose. As already mentioned, two sera for unknown reasons did not show positive readings for agglutinins for a long time. Precipitins were produced very readily in all subjects.

Complement-fixation tests were attempted, but difficulty in the preparation of a suitable antigen delayed this investigation. The mouse-protection experiments were tried, but the mouse is not very suitable as a test animal for streptococci of the viridans group. Even with mucin such large numbers of

organisms are required to kill that another host has to be employed in order properly to assess the protective properties of these presumably immune sera.   Investigations in this direction are in progress.

TABLE 3
Results of precipitation experiments in sera of 56 subjects immunized with a polyvalent vaccine of Streptococcus sanguis

NO. OF SUBJECTS TESTED	WEEKS AFTER 1ST INJECTION	NO. SUBJECTS WITH NEG. RESPONSE	NO. OF SUBJECTS WITH POSITIVE RESPONSE IN SERUM DILUTIONS					
			1:10	1:20	1:30	1:40	1:60	1:160
10	½	6		3		1		
50	2	13	14	11	2	9	1	
3	5	2						1
2	9				1		1	
6	13				4	1	1	
20	15	5		3	7	4	1	
16	17		7	5	3	1		
19	19		3	8	8			
15	21		5	4	3	3		
25	23		2	13	8	2		
20	25		6	8	3	3		

TABLE 4
Comparative agglutination and precipitation titers in sera of 15 subjects immunized with a polyvalent vaccine of Streptococcus sanguis

WEEKS AFTER ONSET OF IMMUNIZATION

SUBJECT	0		2		17		21		25	
	Aggl.	Precip.	Aggl.	Precip.	Aggl.	Precip.	Aggl.	Precip.	Aggl.	Precip.
E. S.	0	0	1:20	1:10	1:20	1:30	1:80	1:30	1:160	1:10
S. S...	0	0	0	0	1:10	1:10	1:40	1:20	1:40	1:20
Sa. S......	0	0	1:10	1:10	1:20	1:10	1:40	1:40	1:80	1:40
L. R... ...	0	0	1:10	1:10	1:20	1:10	1:80	1:40	1:80	1:20
I. K........	0	0	0	1:40	1:40	1:10	1:80	1:30	1:40	1:30
J. H.	0	0	0	0	0	1:30	1:80	1:20	1:40	1:60
T. G.......	0	0	0	1:10	1:20	1:20	1:40	1:10	1:80	1:40
C. C.	0	0	0	0	1:80	1:20	1:160	1:20	1:80	1:20
M. C.	0	0	0	1:20	1:20	1:20	1:20	1:20	1:80	1:20
A. B.......	0	0	1:10	1:20	1:40	1:30	1:160	1:40	1:40	1:20
M. A.......	0	0	1:10	0	1:10	1:20	1:80	1:40	1:160	1:40
M. T.......	0	0	0	1:40	0	1:10	1:20	1:10	1:20	1:10
F. W.......	0	0	0	0	1:10	1:10	1:40	1:10	1:40	1:10
I. S........	0	0	0	1:10	0	1:20			1:80	1:40
A. T........	0	0	1:10	1:30	1:160	1:40	1:160	1:20	1:160	1:30

SUMMARY

Fifty-six patients who have recovered from subacute bacterial endocarditis were immunized with a polyvalent vaccine of *Streptococcus sanguis* (*Streptococcus* s.b.e.).   This is part of a comprehensive prophylactic program to prevent re-

currences in these susceptible individuals. It was hoped, in any event, to prevent infection with *Streptococcus sanguis*, which has proved to be responsible for most of our treatment failures in this disease. The inoculated subjects responded readily to the polyvalent vaccine with the formation of agglutinins and precipitins. Although it is premature to draw any conclusion, it is encouraging that none of these subjects suffered a recurrence of the disease during the course of this 7 months' study.

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STUDIES ON THE BEHAVIOR OF FUNGI IN THE PRESENCE OF RADIOACTIVE ISOTOPES[1]

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Early in 1946 it was found by the authors that certain shipments of radioactive isotopes (P^{32} and I^{131}) were contaminated with fungus spores. At that time these spores were observed under the microscope and cultures of them were identified. As a result of this observation, the authors decided to test the sensitivity of numerous stock cultures of fungi available in this laboratory. The preliminary results of this work have been published (1948). Since that time we have repeated and expanded the work and altered the technique originally used.

MATERIALS AND METHODS

In this study 43 fungi were exposed to radioactive iodine, radioactive phosphorus, and high voltage X-ray. The cultures were grown on Sabouraud's medium until there was a good growth and spores were visible microscopically. Enough sterile saline was added to each tube to bring the water level to the top of the agar slant—approximately 3 to 5 ml. The cultures were taken to the isotope laboratory where 20 microcuries of radioactive phosphorus or iodine were added to each tube.[2] They were left in the isotope laboratory at room temperature for 48 hours; subcultures were then made in the bacteriological laboratory and incubated at room temperature. Cultures of the same fungi were exposed to an equivalent amount of high voltage X-ray (1,100 Roentgen units) and then subcultured. Table 1 shows the effects on the growth of the fungi as compared with the growth of control cultures.

Because distance and adequate filtering are the important factors in protecting the worker from the beta rays emitted by radioactive phosphorus and the gamma rays emitted by radioactive iodine, the workers wore heavy rubber gloves and lead-impregnated aprons, and the isotope solutions were kept behind a lead shield. The solutions were dispensed from pipettes with a special isotope-dispensing bulb attached, thereby eliminating the necessity of pipetting the radioactive solutions with the mouth. It was found to be necessary that two people negotiate the transfer of the radioactive isotopes to the culture tubes. The calculation of the dosage, the pipetting, and the transferring of the radioactive isotopes to the culture tubes were done by a physicist. A second person manipulated the culture tubes with a pair of tongs during the inoculation. The tubes were exposed to the radioactive solutions for 48 hours in the isotope laboratory.

TABLE 1
Effect of radiations on fungi

ORGANISM	GROWTH AFTER EXPOSURE TO		
	X-RAY	P³²	I¹³¹
1. *Actinomyces asteroides*	++	++	++
2. *Alternaria*	++++	+++	+++
3. *Aspergillus*	++++	++++	++++
4. *Aspergillus niger*	++++	++++	+++
5. *Blastomyces brasiliensis*	0	+++	+++
6. *Blastomyces dermatitidis*	+	+++	+++
7. *Candida albicans*	+++	++++	++++
8. *Cephalosporium*	+++	++++	++++
9. *Coccidioides immitis*	++	++	++++
10. *Cryptococcus neoformans*	++++	+++	++++
11. *Epidermophyton floccosum*	0	++++	++
12. *Fusarium*	++++	++++	++++
13. *Geotrichum*	0	++++	+++
14. *Gliocladium*	++++	++++	++++
15. *Helminthosporium*	++++	++++	+++
16. *Histoplasma capsulatum*	++	++++	+
17. *Hormodendrum*	++++	++++	++++
18. *Hormodendrum compactum*	++	+	++
19. *Hormodendrum pedrosoi*	0	+++	++
20. *Microsporum audouini*	0	++++	++
21. *Microsporum canis*	+++	++++	+++
22. *Microsporum gypseum*	0	++++	++++
23. *Microsporum lanosum*	++++	+++	++
24. *Monosporium apiospermum*	++++	+++	+++
25. *Mucor*	++++	++++	++++
26. *Nigrospora*	0	++++	++
27. *Oospora*	++++	++++	++++
28. *Paecilomyces*	++++	++++	+++
29. *Penicillin*	++++	++++	++++
30. *Phialophora verrucosa*	0	++++	+
31. *Rhizopus*	+	++++	+
32. *Rhodotorula*	+++	+++	+++
33. *Scopulariopsis*	+++	++++	+++
34. *Sporotrichum schenckii*	0	++++	++++
35. *Streptomyces*	+++	+++	++++
36. *Syncephalastrum*	++++	++++	++++
37. *Trichoderma*	+++	++++	++++
38. *Trichophyton mentagrophytes*	+++	+++	++
39. *Trichophyton rubrum*	0	+++	+++
40. *Trichophyton schoenleini*	++	++++	++
41. *Trichophyton violaceum*	0	++++	+++
42. *Verticillium*	+++	+++	++

The + signs indicate the amount of growth as compared with growth of control cultures. The amount of growth was graded from 0 (no growth) to ++++ (normal growth).

The racks holding the cultures were taken to the bacteriological laboratory on a metal tray and placed behind a protective shield. Sterile 12-inch drawn-out
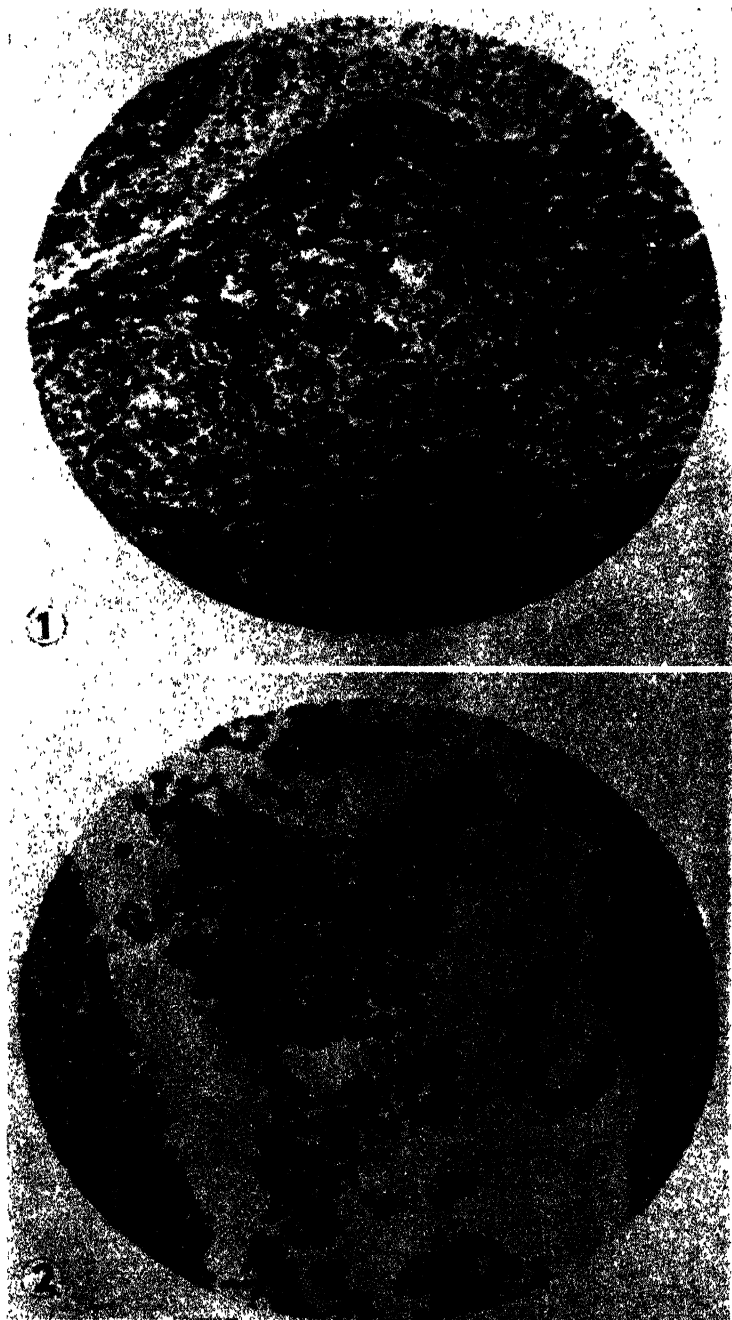
Figure 1. A section of dog liver showing several miliary abscesses, one with an organism, *Blastomyces dermatitidis*, in the center. × 200. H and E and gentian violet stain.

Figure 2. Section through a small bronchus of the same animal, showing purulent exudate which contains several *Blastomyces dermatitidis* organisms. × 200. H and E and gentian violet stain.

Figure 3. (a) A *Fusarium* colony after a 48-hour exposure to 20 microcuries of I[131]. × 125. Dilute methylene blue. (b) Autoradiograph of (a). × 125.

Figure 4. (a) A *Fusarium* colony after a 48-hour exposure to 100 microcuries of P[32]. × 125.   Dilute methylene blue.   (b) Autoradiograph of (a).   × 125.

glass pipettes with rubber bulbs attached were used to agitate the solution in the test tubes until suspended particles of spores and mycelia were visible; several

drops of this suspension were withdrawn with the pipette and inoculated on Sabouraud's slants. During this procedure the workers wore heavy rubber gloves and rubber aprons. Again one person handled the pipetting and a second handled the subcultures. Following each step in the procedure, Geiger counts were done on the laboratories and equipment used and on the personnel involved. The cultures receiving the X-ray were handled in the routine manner without radioactive precautions.

RESULTS

Since the amounts of P^{32}, I^{131}, and X-ray used in this experiment were not lethal and no gross morphological changes in the fungi were noted, a pathogen and a nonpathogen, after exposure to the radioactive solutions, were inoculated intravenously into animals to check any alteration in pathogenicity. *Alternaria*, the nonpathogen, could be followed with the Geiger counter after injection; when the animal was sacrificed after 15 days no lesions were found and the organism could not be cultured from post-mortem material. The animals receiving *Blastomyces dermatitidis*, the pathogen, died in 24 days or less of generalized blastomycosis. The distribution of these organism could also be followed, after injection, with a Geiger counter, and positive cultures of *B. dermatitidis* were obtained from the post-mortem material. (See figures 1 and 2 for photomicrographs of lesions.) The cultures used for injection were filtered through a Seitz filter and washed until the filtrate[3] was radionegative to the Geiger counter. The radioactive spores and mycelia on the filter were resuspended in sterile physiological saline before injection. Preparations made of this material and of *Fusarium* were placed on unexposed lantern slides and autoradiographs obtained, showing that the fungi themselves were radioactive. (See figures 3 and 4.) It was not possible to obtain autoradiographs of single spores or mycelia because the silver granules in the emulsion were too large and there was a considerable amount of scattering. This technique is being studied further and developed.

SUMMARY

A procedure for handling cultures containing radioactive materials is described.

Numerous pathogenic and nonpathogenic fungi survived exposure to radioactive phosphorus, iodine, and high voltage X-ray without alteration in their morphology or pathogenicity.

Solutions of radioactive iodine and phosphorus are not self-sterilizing.

Several autoradiographs of fungi are presented.

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PEARSON, I. A., HAMMER, J. M., AND HILL, E. J. 1948 The behavior of fungi in the presence of radioactive tracers. Harper Hosp. Bull., **6**, 46–55.

[3] A filtrate that gave not more than 10 C/M/ml was considered to be radionegative.

THE GROWTH OF T2 VIRUS ON ULTRAVIOLET-KILLED HOST CELLS[1,2]

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Until recently the general impression has been that a virus requires a complete living host cell for its multiplication, since cells killed by heat or certain chemicals not only fail to support virus growth but inactivate any virus which becomes attached to them. Since such agents as these doubtless produce rather general havoc in a cell, destroying the balanced functional properties of many of the constituents, it seemed desirable to investigate the possibility that a virus might be able to utilize for its multiplication the cellular apparatus of host cells that had been damaged in a more discrete manner.

I have studied the interactions of T2 virus with host cells (*Escherichia coli*, strain B) that have been irradiated with ultraviolet light. Here the primary damage to the cell, being produced by the absorption of individual quanta of radiant energy, is probably limited to molecular dimensions at or near the sites of absorption, but scattered throughout the volume of the cell in accordance with the distribution of absorbing material. It was found that cells that had been killed many times over by ultraviolet irradiation were still capable of supporting virus growth.

MATERIALS AND METHODS

The bacterial virus T2 (virus gamma of Luria, Delbrück, and Anderson, 1943) and strain B of *Escherichia coli* were used in the present study. A synthetic medium (Anderson, 1945) containing ammonium lactate as the sole source of carbon and nitrogen was used for liquid culture since it is transparent to the ultraviolet light used and supported bacterial growth up to 1 g per liter and T2 virus growth on these bacteria to 5×10^{10} active particles per ml. One per cent agar in this medium was employed as a solid medium for both bacterial assay by the colony-counting method and virus assay by the plaque-counting method of Delbrück and Luria (1942).

A General Electric Company type H-4 mercury lamp with the outer glass shell removed served as an intense source of ultraviolet light. Cultures were irradiated in a quartz test tube of 1-cm internal diameter, which in turn was placed inside another quartz test tube of 2.5-cm internal diameter. The latter tube was filled with ethyl alcohol to filter out wave lengths shorter than 2,200 A.

This assembly was placed 5 inches from the lamp. In all the experiments the liquid cultures were stirred continuously by aeration during irradiation. Short exposures to the ultraviolet were timed by a 180° sector driven by a synchronous motor that made one revolution per second. The motor was actuated by a switch that caused it to make one rotation and then stop in a position to interrupt the light beam. Each time the switch was closed the specimen thus received one-half second of irradiation.

Much less intense, but monochromatic, ultraviolet light was obtained using an H-4 lamp with the outer glass shell removed as the source and a quartz monochromator to select individual lines in the mercury spectrum.

EXPERIMENTAL RESULTS

In preliminary experiments the host was exposed to relatively weak monochromatic radiation at λ 2,537 obtained with the monochromator. This gave a first-order rate of killing of the cells as determined by colony counts, 10 minutes of irradiation being required to reduce the colony count to 10 per cent of its initial value. Virus T2 was added to cells exposed for 0, 20, 40, 60, and 80 minutes; after 10 minutes had been allowed for adsorption in each case, the mixtures were assayed for the number of plaque-forming particles. An excess of fresh, unirradiated bacteria was used for pouring the plates. It was found that the plaque count was not reduced appreciably after the exposure of the virus to bacterial suspensions that had been irradiated for as much as 80 minutes. Either the virus had not been adsorbed on the damaged cells or it had been adsorbed and had not been inactivated. To obtain data on the doses required for the production of virus-inactivating cultures we turned to the more intense direct irradiation with the H-4 lamp.

With the H-4 lamp the killing of the bacteria again followed the first-order equation (Gowen, 1936):

$$N/No = e^{-kt} \tag{1}$$

Here No is the original number of colony-forming organisms per ml and N is the number able to form colonies after an exposure of t seconds. In the succeeding discussion we shall term the product kt the number of "lethal doses." The rate constant k was quite constant, varying between 0.75 and 0.59 sec^{-1} over a period of months.

In experiments modeled after that outlined above it was found, as illustrated in figure 1, that with continued irradiation host suspensions slowly acquire the ability to inactivate added virus and then, as irradiation is continued, this destructive ability is gradually lost. From these results it seemed likely that (besides killing the cells) irradiation affected the cells' interaction with virus in two ways: first it rendered them unsuited for virus multiplication, so that the added virus, being adsorbed, was inactivated; and, secondly, the radiation more slowly destroyed the cells' ability to adsorb virus so that virus added to the most severely damaged cells was able to survive.

It was now necessary to find out whether killed cells had actually been able to

adsorb virus particles.   For this purpose adsorption mixtures of virus and irradiated cultures were centrifuged at 4,000 rpm for 5 minutes, a treatment sufficient to throw down the bacteria with adsorbed virus, but not sufficient to sediment the much smaller free virus particles.   Assays made of the supernatant
liquids then yielded the concentrations of free and active virus particles, whereas
assays of the resuspended sediments provided estimates of the concentrations
of *adsorbed* virus particles that were able to form plaques.   In this manner the
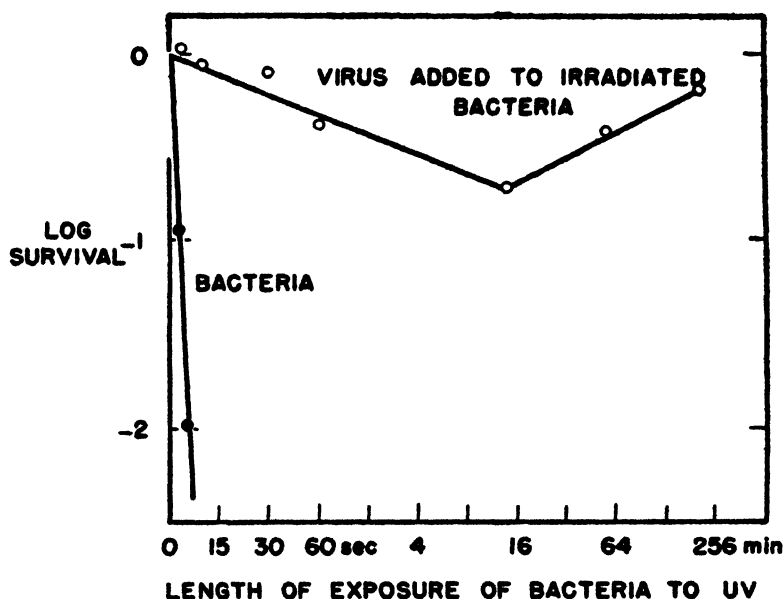
Figure 1. Log survival of bacteria exposed to the intense radiation from the H-4 lamp
(solid circles) and of T2 virus added to the bacteria after irradiation (open circles).   The
bacteria in ammonium lactate medium had been aerated at 37 C for 2 hours and had reached
a concentration of 4×10^8 cells per ml when an aliquot was irradiated.   One-tenth-ml samples were removed at early intervals for colony counts, and at later intervals 0.9-ml samples
were removed for virus-inactivation experiments.   About 2×10^9 T2 particles in 0.1 ml of
the synthetic medium were added to each of the samples, and after 10 minutes had been
allowed for adsorption the mixtures were assayed for plaque-forming particles by plating
0.1-ml samples with an excess of normal bacteria.   Note that the time scale in the figure
is expanded for short exposures and compressed for long exposures.   The bacteria were
rapidly killed, but the bacterial suspension slowly acquired and then gradually lost the
capacity to inactivate the virus as the exposure was prolonged.

data presented in figure 2 were obtained.   It is seen that appreciable numbers
of plaques were produced by sedimented bacteria that before infection had
received 30 lethal doses (50 seconds) of irradiation.   After such treatment only
one in 10^{13} cells (more than were used in the entire experiment) would have been
able to produce a colony.   We conclude that UV-killed cells can not only adsorb
virus but, after plating, cells which have been killed and then infected are able
to liberate active virus particles and so initiate the formation of plaques.

As far as one can judge from the foregoing experiments the formation of plaques

from these damaged and infected cells could have been due to the direct release of the adsorbed virus particles after they were plated or to virus growth and lysis of the infected cells with the liberation of many new virus particles.

To see whether virus multiplication had occurred, the average number of virus particles liberated per damaged bacterium (burst size) was determined in experiments following the procedure outlined in table 1. It is seen from the results given there that an average of 18 virus particles were liberated from cells
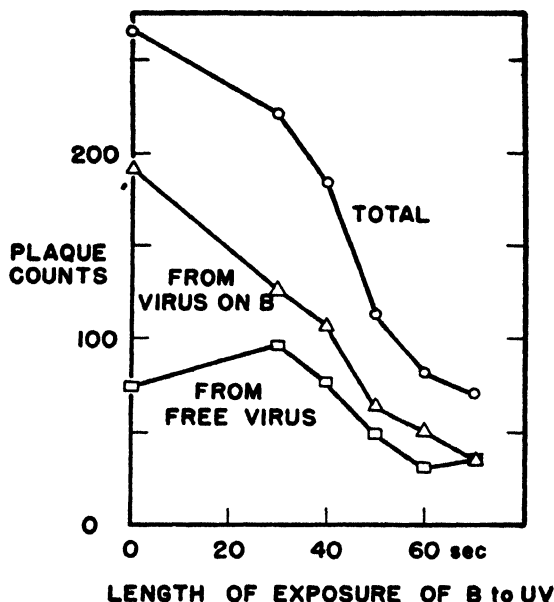
Figure 2. Total plaque counts (circles), plaques from virus adsorbed on irradiated bacteria (triangles), and plaques from free virus (squares). The aerated bacterial culture had grown to 5×10^8 colony-forming cells per ml when a sample was irradiated with light from the H-4 lamp. Samples irradiated for 0, 4, 8, and 12 seconds were assayed by colony counts for the survival of the bacteria. Samples of 0.9 ml were removed after exposures of 0, 30, 40, 50, 60, and 70 seconds, and 2.7×10^3 T2 particles in 0.1 ml were added to each. After 10 minutes had been allowed for adsorption the bacteria and adsorbed virus were centrifuged down leaving the free virus in the supernatant. The plaques formed by 0.1 ml of the supernatant (free virus), that from 0.1 ml of the sediment resuspended in 1.0 ml (adsorbed virus), and the total counts are plotted in the figure against the length of exposure of the host cells. The concentrations of colony-forming cells were as follows:

Exposure (seconds)	0	4	8	12
Colony-forming cells per ml	5×10^8	6×10^7	6×10^6	5×10^5

that could liberate *some* virus after receiving 9 lethal doses of irradiation ($kt = 9$). The unexposed control cells released 37 particles on the average. The results of many such experiments in which various doses of irradiation were given the bacteria before infection are plotted in figure 3. It is seen that the burst sizes from those cells that were able to liberate some active virus decreased as the pre-infection damage to the cells was increased. Otherwise the curves obtained in the burst size determinations appeared to be normal.

We conclude that T2 bacteriophage is able to multiply on host cells that have

TABLE 1

The burst size of virus T2 on control and on irradiated bacteria

Bacteria from a 3½-hr, 37 C, aerated culture in a synthetic medium containing 1.4×10^8 cells per ml were given 9 lethal doses of irradiation from the H-4 lamp. Virus was then added to the irradiated bacteria and to the unexposed control, and after 5 minutes had been allowed for adsorption, the suspensions were greatly diluted in synthetic medium and incubated with aeration at 37 C. The virus titers were determined at intervals as in the work of Delbrück and Luria (1942).

	CONTROL BACTERIA	IRRADIATED BACTERIA
Adsorption time	5 min	5 min
Total bacteria/ml after dilution	6,400	6,400
Virus particles/ml	5,500	5,500
Initial virus titer	5,300	5,800
Initial unabsorbed virus/ml*	1,800	2,500
Initial multiplicity of infection $= \dfrac{\text{Initial virus titer} - \text{initial unadsorbed virus}}{\text{total bacteria}}$	0.58	0.47
Final virus titer	130,000	62,000
Burst size $= \dfrac{\text{Final virus titer} - \text{initial unadsorbed virus}}{\text{Initial virus titer} - \text{initial unadsorbed virus}}$	37	18

* Determined from assays of supernatants of the diluted mixture.
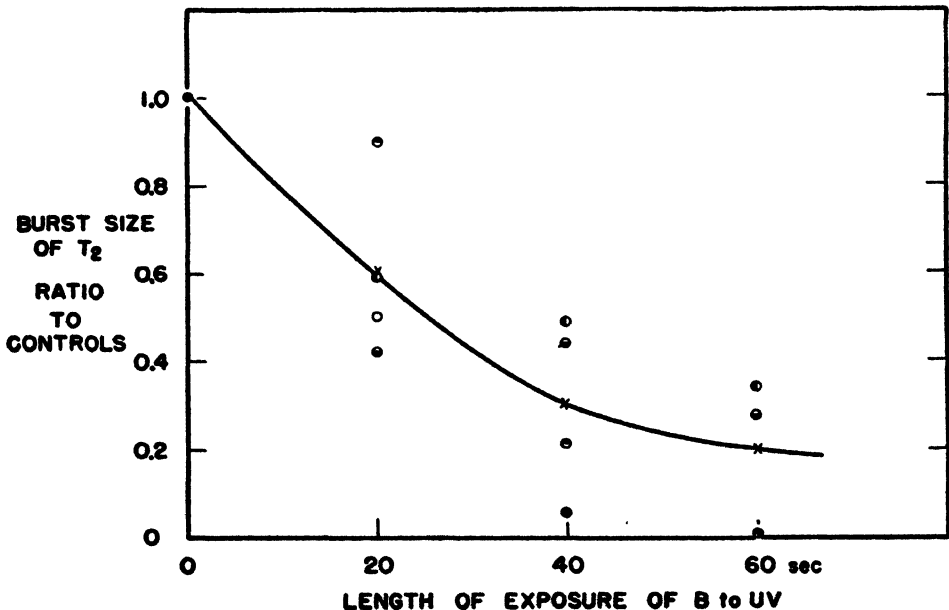
Figure 3. The ratios of the burst sizes of T2 virus on irradiated bacteria to burst sizes on control bacteria in a series of experiments patterned after that of table 1. It is seen that the burst sizes decrease progressively with increasing exposures of the bacteria to ultraviolet irradiation. The data for each experiment are given individual circle symbols in the graph, but the curve has been drawn through the average values for each exposure that are indicated by crosses.

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received many lethal doses of irradiation but that irradiation damage decreases the average number of virus particles liberated per cell.

DISCUSSION

It seems clear from the foregoing results that the complete moiety of cellular constituents required for continued multiplication of the host to form a colony is not required for T2 proliferation and the release of daughter particles. A similar conclusion might be drawn from the observation of Rouyer and Latarjet (1946) that bacteriophage C16 will multiply on its host, "bacille paradysenterique Y6R," after the latter has received many lethal doses of X-irradiation. Other lines of evidence point to the same conclusion. For example, Spizizen (1943) has observed the multiplication of a bacterial virus on host cells in media containing metabolic poisons or limitations of nutrient such that the cells could not multiply. In these three cases bacteriophages have been observed to multiply under conditions that are lethal to the host cell.

Mutations of host cells to resistance set up conditions under which the mutant cells are viable but fail to support virus growth. Studies of such cells have thus provided additional clues to the relation of virus requirements to cellular constituents. Certain of these mutations in *E. coli*, strain B, leave the cell nutritionally deficient, presumably through the deletion of certain enzyme systems (E. H. Anderson, 1944, 1946). The altered host thus becomes resistant to one or more of the viruses active on the parent strain, *but remains sensitive to the others.* E. H. Anderson (1944) infers from this that all of the original cellular apparatus is not required for the multiplication of the viruses. Still the foregoing mutant cells are viable; if supplied with suitable nutrients theyc an multiply indefinitely to form colonies. Therefore, all of the cellular apparatus of the parent cell is not required for viability either.

We are thus led to view the cell as a group of functionally co-ordinated elements. One subgroup of these elements is essential to the cell's viability. Other subgroups, possibly unique for each virus type but in general different from that required for viability, are essential to the multiplication of the viruses active on the cell. The elimination of an element by mutation or otherwise might lead to a cell that is resistant to the viruses whose subgroups contain that element, or to a nonviable cell if the element is contained in the subgroup essential to cellular multiplication.

Work with resistant cultures is limited to deletions of the viable type in which the deleted element is not a member of the viability subgroup, but it has the advantage that clues to the nature of the deleted element can be found in favorable cases.

The growth of virus on radiation-damaged cells shows that many of the elements in the viability subgroup are not contained in the virus subgroup. Until agents of known specificities are used, however, there will be little chance of characterizing the elements of the viability subgroup.

Nevertheless, the inactivation of virus by heavily damaged cells does tell us something of the nature of the virus-host complex. From figures 1 and 2

it may be seen that the proportion of particles inactivated by the bacterial suspensions increases with increasing irradiation of the cultures. It seems likely that many of these particles are irreversibly adsorbed on bacteria that have been so severely damaged that they fail to support virus multiplication. Without adsorption it is difficult to explain the summary lysis of heavily irradiated host cells by T2 virus (Anderson, 1945) or by T4 virus activated by L-tryptophan (Anderson, 1948). Heat killing or more severe chemical treatment of the host cells results in a cell that similarly inactivates the adsorbed virus. It would appear that once a virus particle is adsorbed on the host cell it is committed to a set course of action that normally would lead to virus multiplication and lysis of the host. If the cell has been damaged sufficiently, the course is somehow blocked; the virus can neither multiply nor free itself to infect other host cells. A strikingly analogous effect is observed if irradiated T2 virus is allowed to react with normal host cells (Luria and Delbrück, 1942). The irradiated virus is unable to multiply, but when adsorbed on the host it apparently interacts irreversibly with constituents essential to the host cell and so prevents its multiplication. The present results suggest the converse; namely, that certain constituents of the host cell whose structures have been altered by the absorption of ultraviolet light can inactivate virus elements which they engage in much the same manner in which certain molecules whose structures differ but slightly from those of normal substrates are supposed to inactivate enzyme molecules which they engage.

However, more than the simple inactivation of the infecting virus particle is involved at these doses of irradiation, for irradiation of the host reduced the burst size of T2. This reduction in the number of virus particles liberated from those cells that produce *some* virus may be due to a decrease in the amount of certain intracellular nutrients required for multiplication of the virus (Delbrück, 1945). However, a reduction in burst size by a factor of three or more in going from nutrient medium (burst size = 120; Delbrück, 1946) to ammonium lactate medium (burst size = 30 to 40) leads to no measurable reduction in the chances of survival of adsorbed virus. Since in our experiments the reduction of the chances of survival of adsorbed virus runs roughly parallel to the reduction in burst size, it seems possible that similar mechanisms may be responsible for both effects.

SUMMARY

Cultures of *Escherichia coli*, strain B, were irradiated with ultraviolet light and the rate of killing of the cells was determined. The suitability of the irradiated cells as hosts for the bacteriophage T2 was then followed. It was found that T2 could multiply on the cells after they had received many lethal doses of irradiation. However, increasing the preinfection dose of irradiation slowly reduced both the chances of survival of adsorbed virus and the average number of active T2 particles liberated from those damaged cells that released *some* active virus. Still more severe irradiation of the host eventually destroyed its ability to adsorb and inactivate virus.

It is postulated that in its proliferation the virus conjugates with a moiety of cellular elements that is distinct from the moiety required by the cell for its unlimited division. The virus moiety is much less sensitive to irradiation than the cellular moiety. Damaged elements in the virus moiety are supposed to inactivate virus elements which they engage in a manner similar to that in which analogues of normal substrates are supposed to inactivate enzyme molecules which they engage.

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ANTIGENIC RELATIONSHIPS OF 765 PARACOLOBACTRUM INTERMEDIUM CULTURES

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In a study of the antigenic relationships of *Paracolobactrum*,[1] Stuart, Wheeler, *et al.* (1943) found that 48 per cent of 140 *Paracolobactrum aerogenoides* strains were identical or closely related to one or another of 8 of the strains used to produce antiserums. Fifty-three per cent of 40 *Paracolobactrum intermedium* cultures were identical with one or another of 6 strains used to prepare antiserums; and 67 per cent of 223 *Paracolobactrum coliforme* strains were identical or closely related to one or another of 13 strains used to produce antiserums. These results indicated that the investigation should be continued.

In 3 years more than one thousand additional cultures comprising all three groups were investigated. At this time certain conclusions could be drawn. As the number of *P. coliforme* strains increased, the percentage of cultures identified by the original 13 antiserums decreased rapidly. Sixty-five per cent of the first 250 strains isolated were identified in these serums. But only 46 per cent of the next 250 strains, and 19 per cent of the last 250 strains could be identified. A similar situation was encountered in isolating and identifying various strains of *P. aerogenoides.* Biochemical type 32011 (Stuart and Rustigian, 1943), an exception to this observation, was encountered throughout the investigation.

In addition, it was observed that over a certain time interval a given serological type was isolated consistently from any one institution or group of institutions. At a later period, however, all types encountered, although antigenically identical or closely related to one another, differed markedly from those isolated over the previous time interval. The limits of each period varied with the type and with its source. There was also a certain degree of overlapping among the types involved. For example, all 100 strains of *P. coliforme* (31611) were isolated in Providence, Rhode Island, or the immediate vicinity, between 1939 and 1944. Since that time this type seems to have disappeared entirely. Twenty-three strains of type 111 came from a single institution over a period of 3 years, but

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[1] Borman, Stuart, and Wheeler (1944) proposed the term "*Paracolobactrum*" for that large group of organisms possessing physiological and serological characteristics common to coliform and *Salmonella* bacteria. It was further suggested that the *Paracolobactrum* be divided into three sections or groups, *P. aerogenoides, P. intermedium*, and *P. coliforme.* For a more detailed description of these terms see the sixth edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Hitchens, 1948). In order to correlate the terms used in the present work with those of the past (Stuart, Wheeler, *et al.*, 1943; and other publications), *Paracolobactrum* would be interpreted as paracolon; *P. aerogenoides* as paracolon *Aerobacter; P. intermedium* as paracolon intermediate; and *P. coliforme* as paracolon *Escherichia.*

this type was not encountered again after that period. On the other hand, a single biochemical type occasionally persisted in one institution over periods of several years. In such instances, however, a succession of serological types was usually encountered. This "temporal and topographical antigenic continuity" was found also in *Escherichia coli* (Stuart and Carpenter, 1948).

The number of new *P. intermedium* isolates was small, but the percentage identified in 5 of the 6 original antiserums remained high. It was decided, therefore, to concentrate on this group to the exclusion of the other aerogenic *Paracolobactrum* groups.

All *Paracolobactrum* cultures isolated in the Florida State Public Health Laboratories and the *Paracolobactrum* cultures received at Brown University from state and municipal laboratories as far west as California totaled 1,957 in the year 1946–1947. These cultures were isolated in the various laboratories on *Salmonella-Shigella*, Wilson-Blair, brilliant green, and eosin-methylene-blue agar. They were replated on eosin-methylene-blue agar, the least inhibitory of these four mediums, and a well-isolated colony was transplanted to an agar slant. Strains selected for serological study produced acid and gas rapidly in glucose, maltose, and mannitol, almost always attached lactose slowly, seldom fermented sucrose, and frequently fermented salicin slowly. They were motile, grew on citrate agar, and produced hydrogen sulfide. They failed to produce indole or acetylmethylcarbinol and were urea-negative in the medium of Rustigian and Stuart (1941). A few strains producing acid but not gas in lactose in 24 hours were included in this investigation.

Of the 1,957 cultures obtained, 765 or 39 per cent were selected for further study and were classified as *P. intermedium*. About 40 per cent of all *Paracolobactrum* cultures isolated in Florida were *P. intermedium*, but only 5 to 10 per cent of *Paracolobactrum* from other areas were *P. intermedium*. These percentages have remained fairly consistent in the areas involved over periods of from 2 to 3 years and in the Rhode Island area for 10 years.

In general the biochemical reactions of these *P. intermedium* cultures were quite consistent. An occasional strain failed to ferment lactose; many strains were anaerogenic in lactose and some required 60 days to attack this carbohydrate. Salicin, when positive, was attacked more slowly than lactose. The great majority, however, produced acid or acid and a bubble of gas in lactose in from 3 to 9 days and in salicin in 1 to 3 weeks. Large gas volumes, 20 to 40 per cent, were seldom encountered in lactose and salicin. Sucrose was fermented slowly by some strains. Several strains produced variants that utilized citrate slowly or not at all. A few strains yielded variants that failed to produce H$_2$S or produced it slowly. Two strains produced indole-positive variants, and another, an acetylmethylcarbinol-positive variant. There was good antigenic agreement among strains producing acid rapidly in lactose, among strains failing to ferment lactose, and among strains fermenting sucrose. However, there were sufficient serological exceptions to render biochemical classification impractical. Moreover, some lactose-negative strains were trained to ferment lactose. Occasionally sucrose-negative variants were obtained from sucrose-positive parents.

A large number of strains positive in salicin when freshly isolated were negative on subsequent tests. For the most part, plating such cultures on EMB agar containing salicin instead of lactose failed to establish salicin-positive variants. A similar situation was found for *Shigella alkalescens* with respect to sucrose (Stuart and Rustigian, 1944). When definite serological types were established, additional biochemical reactions were determined on representative numbers of the different types. Table 1 shows the biochemical reactions of the 12 most common types.

TABLE 1

Biochemical reactions of the 12 most common serological types of Paracolobactrum intermedium encountered in the present work

TYPE	NUMBER STRAINS	GLUCOSE GALACTOSE MALTOSE MANNITOL RHAMNOSE ARABINOSE XYLOSE SORBITOL	INOSITOL ADONITOL	LACTOSE	SUCROSE	SALICIN	DULCITOL	RAFFINOSE	TARTRATE	CITRATE H₂S MOTILITY	INDOLE V-P UREA LITMUS MILK
5883	11	+	−	+•	−	+•	+	−	*W*	+	−
9466	21	+	−	+•	−	±•	+	−	−	+	−
13304	13	+	−	+•	±•	+•	+	−	−	+	−
20157	9	+	−	+•	−	−	+	−	−	+	−
20158	99	+	−	+•	−	+•	±	−	*W*	+	−
20565	65	+	−	+•	−	+•	+	−	−	+	−
8-519	18	+	−	+•	±•	±•	+	−	−	+	−
8624	18	+	−	+•	−	−	+	−	−	+	−
1805	67	+	−	+•	−	+•	+	−	+	+	−
18800	13	+	−	+•	−	+•	−	−	+	+	−
4440	26	+	−	+•	−	±•	±	−	−	+	−
5099	13	+	−	+	−	+•	+	+	+	+	−

+ = rapid production of acid and gas.
+• = slow production of acid or acid and gas.
± = some strains positive and others negative.
W = some strains weakly positive and others negative.

As fast as new strains were isolated they were tested in 5 *P. intermedium* antiserums prepared in the previous work. Serial dilutions of the antiserums ranging from 40 to 40,960 were prepared. Living saline suspensions of 16- to 24-hour agar transplants served as antigens. Results were recorded after 2 hours in a 37 C water bath and again after holding overnight in a 55 C water bath. Certain advantages of this incubation system over the conventional 2 hours at 50 C followed by an overnight period at 2 C have been discussed in another report (Stuart and Carpenter, 1948). Of the first 150 strains tested, many gave strong reactions in antiserum 14011 and a few strains in antiserum 12611. An occasional strain reacted in 2 other serums but none reacted in the fifth serum. Upon adsorption no strain was found to be antigenically identical with any of the 5 used to produce antiserums. From the results of these tests 15 cultures were selected for the preparation of additional antiserums. After considerable exploratory

work 9 antiserums giving the highest titers with the greatest number of strains were selected as "basic types." Only one of the 5 original antiserums (14011) was included in the nine. Each of the 765 cultures was tested in the 9 basic antiserums. In these tests 0.1 ml of a saline suspension of living antigen was added to 0.5-ml portions of 80, 640, and 5,120 dilutions of each antiserum. Tubes were incubated as previously described.

Many definite antigenic patterns were established by the 9 basic antiserums. Table 2 shows some of the more common patterns. All strains having an antigenic pattern comparable to 5883, 9466, 13304, and 20158, etc., were used to adsorb the antiserums of these respective strains. Two hundred forty-one strains were identified with the 9 basic antiserums. Other antigenic patterns were studied, and, when a sufficient number of strains showed a comparable pattern, an antiserum was prepared from one of the strains. In this way a total of 34 antiserums was used to study this group of *P. intermedium* organisms.

Of the 765 cultures, 453 or 59 per cent were antigenically identical with one or another of the 34 cultures used to produce antiserums. Strains were called antigenically identical when they agglutinated to titer in an antiserum and removed all agglutinins upon adsorption. They were considered closely related when they agglutinated to titer and markedly reduced the titer upon adsorption. The most commonly encountered type was 20158 (table 1) with 99 strains. This type was found in Rhode Island, Florida, California, and elsewhere. Sixty-seven strains of type 1805 (table 1) having the same somatic antigens as 20158 were also widespread. Sixty-one additional strains, comprising 11 different serotypes, possessed somatic antigens related to or identical with those of 20158. Therefore 227 or 52 per cent of the identified strains or 29 per cent of all *P. intermedium* strains had the same or related somatic antigens. On the other hand, 312 strains or 41 per cent of the total could not be identified by any of the 34 antiserums, although many of the 312 strains were closely related to one or another of the 9 basic strains used to produce antiserums. Moreover, despite the fact that 140 strains possessed the antigenic pattern of S-519 (table 2), only 16 were identical with S-519. Five more antiserums were prepared from cultures in this group, but only 50 or 36 per cent of the 140 strains were identified in these 6 antiserums.

Thirty-two strains failed to agglutinate in any of the 9 basic antiserums even when tests were repeated in a dilution of 20. Twenty-five of these constituted the only group that rapidly produced acid in lactose. An antiserum prepared from one of the 25 identified 13 strains; an antiserum prepared from another strain identified 2; and an antiserum from a third identified only its homologous strain.

DISCUSSION

The general antigenic relationships of 765 strains of *P. intermedium* were determined in the present investigation. No attempt was made to set up a diagnostic typing scheme. Typing systems are a great aid in diagnostic bacteriology but if carried too far may impede progress in the field as a whole. Like the Cohn-Koch dogma of monomorphism they tend to create a false impression of security and

TABLE 2

Antigenic pattern of selected Paracolobactrum intermedium strains in the nine basic antiserums

ANTIGEN	TEMP.	5883			7328			9466			13304			14011			20157			20158			20565			S-519		
		80	640	5,120	80	640	5,120	80	640	5,120	80	640	5,120	80	640	5,120	80	640	5,120	80	640	5,120	80	640	5,120	80	640	5,120
5883	37	3	3	3	3	1	−	−			3	3	3	2	−		3	3	3	3	3	3	3	3	1	2	−	
	55	4	4	4	4	2	−	−			−			−			4	4	3	−			4	4	2	−		
9466	37	2	−		3	1	−	3	3	3	3	2		2			3	1	−	−			2			3	2	−
	55	−			2	−	−	4	4	4	−			−			−			−			−			−		
13304	37	3	3	2	3	3	1	−	−		3	3	3	3	3	1	3	3	3	3	3	1	2	−		3	3	3
	55	−			4	4	2	−	−		4	4	4	4	4	2	2			2	−		2	−		4	3	4
20158	37	2	3	3	−			−			3	3	1	−			3	3	3	3	3	3	−			−		
	55	−			−			−			−			−			−			4	4	4	−			−		
8-519	37	−			3	3	2	−			3	3	2	3	3	2	3	1	−	2			−			3	3	
	55	−			4	4	2	−			4	4	2	4	4	3	2	−		−			−			4	4	
1805	37	−			−			−			−	1		−			2	−		3	3	3	2			−		
	55	−			−			−			−	−		−			−			4	4	4	−			−		
4440	37	2	3	2	3	3	2	−			3	3	2	3	3	3	3	3	3	−			3	3	3	2	−	2
	55	4	4	3	4	2	−	−			2	4	2	2	−		4	4	4	−			4	4	3	2	−	3
8624	37	2	−		3	3	1	−			3	3	3	3	3	2	2	−		−			3	3	2	3	3	2
	55	−			4	4	2	−			4	4	3	4	4	3	−			−			−			4	4	2
?	37	3	3	3	3	3	2	−			3	3	3	3	3	3	3	3	3	3	3	2	3	1	−	3	3	2
	55	4	4	3	4	4	2	−			4	4	3	4	4	4	4	4	2	4	3	1	−			4	4	2

4, 3, 2, 1, − = degrees of agglutination from complete to negative.   ? = unidentified strain.

finality.   Studies involving the unrestricted use of single factor serums probably err as much by missing important relationships as the unrestricted use of whole antiserums confuses the issue by uncovering too many unimportant relationships. Gard (1937), Gard and Ericson (1939), Schiff, Bornstein, and Saphra (1941), Wheeler, Stuart, *et al.* (1943), and many others found labeled H and O *Salmonella* antigens in coliform bacteria.   Stuart, Wheeler, *et al.* (1943) found a number of coliform cultures agglutinating to high titer, occasionally to the homologous titer, in whole *Salmonella* antiserums.   These cross reactions were due to unlabeled antigens that in a study of taxonomic relationships could be as important or more important than labeled antigens.

The 34 strains used to produce antiserums, tested in *Salmonella* single factor serums with the slide test, showed no significant cross reactions.

Previous to the present work, Edwards, West, and Bruner (1948) made an intensive study of 32 *P. intermedium* cultures isolated in four outbreaks of gastro-enteritis.   Agglutination and adsorption tests established 4 groups of O and 5 groups of H antigens.   The 32 cultures were divisible into 8 types.   For convenience these investigators called their strains the "Bethesda" group.   Dr. Edwards kindly furnished us with unadsorbed Bethesda antiserums and their homologous cultures.   Agglutination and adsorption tests with the Bethesda cultures and antiserums and the 9 basic cultures and antiserums of the present work revealed that 3 of the 9 basic cultures, including strain 20158, were identical with one or another of the Bethesda cultures.   All of the 6 remaining basic cultures were related antigenically to the Bethesda strains.   Obviously a very high percentage of the 765 cultures studied in the present work belonged to the Bethesda group.

The hope that *P. intermedium* cultures would show marked antigenic continuity irrespective of the time and place of isolation was not realized completely. Only one of the 5 antiserums (14011) prepared in 1939 was of real value in the present work.   Although a number of strains were related antigenically to 14011, only one strain was identical with it.   Among the first 100 *Paracolobactrum* cultures isolated in Florida were 21 *P. intermedium* (type 9466) strains (table 2). Sixteen of the 21 strains came from normal children in one institution and the remainder from different sources.   This type was not encountered again in Florida nor was it found in any other part of the country.   Therefore there is some temporal and topographical antigenic continuity in *P. intermedium*, but it seems much less marked than in the other *Paracolobactrum* groups or the coliform groups.

P. aerogenoides and *P. coliforme* cultures for the most part can be distinguished easily from *Salmonella* by positive Voges-Proskauer and indole reactions, respectively, in 24 hours or less.   Most *P. intermedium* cultures cannot be differentiated from some *Salmonella* species by biochemical reactions for several days or more. The nuisance value of the *P. intermedium* group far exceeds that of the other *Paracolobactrum* groups.   Holding lactose, sucrose, and salicin tubes until one or another is positive may cause prolonged delay in "reporting out" the culture, whereas running any considerable number of these cultures in *Salmonella* typing serums results in a waste of time and expensive typing serums.

Although *P. intermedium* organisms, including the Bethesda group, doubtless can cause acute gastroenteritis of varying duration, on the whole their pathogenicity does not seem very great. For every type isolated from gastroenteritis patients a greater number of the same type, where significant numbers were involved, were isolated from normal individuals in food handler and industrial surveys. The antigens of certain Bethesda types because of their wide distribution and frequent occurrence, if for no other reason, might well be included in some diagnostic typing scheme. How extensive and inclusive such a scheme should be must depend to some degree on further investigations.

ACKNOWLEDGMENT

We are indebted to Dr. K. M. Wheeler, deceased, who tested in single factor *Salmonella* typing serums the 34 *P. intermedium* strains used to produce antiserums in the present work.

CONCLUSIONS

Of 765 strains of *Paracolobactrum intermedium*, 453 or 59 per cent were antigenically identical with one or another of 34 strains used to produce antiserums. Two hundred twenty-seven strains, 52 per cent of the 453 identified or 29 per cent of the total of 765 strains, possessed the same or closely related somatic antigens. Antigenic continuity appears to be much greater in the *Paracolobactrum intermedium* group than in the *Paracolobactrum coliforme* and *Paracolobactrum aerogenoides* groups. Although strains of *P. intermedium* doubtless can cause acute gastroenteritis, the group as a whole does not present a serious public health problem.

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AN UNUSUAL TYPE OF O AGGLUTINATION

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Several years ago in this laboratory an unusual type of agglutination was encountered in an investigation of the serology of *Shigella alkalescens*. On occasions a certain strain of *S. alkalescens* in homologous antiserum after 18 hours at 55 C showed the usual type of O agglutination with heavy compact granules on the bottom of the tube and crystal-clear supernatant fluid. The granules were altered only slightly when the tubes were shaken vigorously. On other occasions, however, this same strain in the same antiserum and under the same conditions of incubation gave an entirely different type of agglutination. The supernatant fluids were somewhat turbid and a soft mass of adherent cells lay on the bottom of the tubes. When the tubes were flirted gently, threads of agglutinated bacteria spiraled upwards. Upon vigorous shaking the threads either broke up into fine visible particles or disintegrated into even finer particles that were invisibly dispersed in the supernatant. If, after the shaking, a test was incubated again at 55 C for several hours, the same type of agglutination occurred. In protocols the first type of agglutination described above was recorded as "O" (granular) and the second "T" (thready). A culture exhibiting the T reaction might agglutinate to the somatic titer of its homologous antiserum or might fall short by one to four dilutions.

Personal communications revealed that other investigators working with enteric bacteria had encountered occasionally "mucoid," "slimy," or "stringy" reactions comparable to the T type of agglutination.

The T type of agglutination was found in a few *Paracolobactrum*, type 29911, *Proteus rettgeri* strains, and in coliform and *Salmonella ballerup* strains with and without Vi antigen. In cultures exhibiting V to W variation, T agglutination was much more pronounced with V forms. Thus T agglutination in some instances may be correlated with the Vi antigen.

Because of the pressure of other investigations and the infrequency of T agglutination the reaction was not studied further although the practice of recording O and T agglutination in protocols was continued. In a recent serological study of 765 strains of *Paracolobactrum intermedium* the T type of agglutination was the rule rather than the exception (Stuart, Galton, and McGann, 1948).

The incubation temperature of agglutination tests was very important in this work. Most *S. alkalescens* strains and some other nonmotile cultures that gave little or no agglutination of any kind after 2 hours at 50 C and 18 hours at 2 C showed perfect O or T agglutination after 2 hours at 37 C and 18 hours at 55 C. Actively motile strains of *P. intermedium*, such as strain 20158 (Stuart, Galton, and McGann, 1948), gave excellent H reactions after 2 hours at 37 C and perfect T reactions after 18 hours at 55 C (table 1, A). These strains, including 20158,

showed good H agglutination after 2 hours at 50 C but little or no T agglutination after 18 hours at 2 C. Since T agglutination aided in typing some strains of *P. intermedium*, the nature of T agglutination was studied.

Saline suspensions of cultures showing T agglutination were heated in a boiling water bath for 2 hours or less; T reactions were eliminated and O agglutination was restored. This would indicate that the substance responsible for T agglutination was thermolabile, in agreement with Felix and Pitt (1934) and many others who, on the same evidence, assumed that the Vi antigen was thermolabile. However, Stuart and Kennedy (1948) showed that heating the

TABLE 1

Agglutination of 20158 antigen in 20158 antiserum

EXPERIMENTAL CONDITIONS	CHARACTER OF ANTIGEN 20158	INCUBATION TEMP. (C)	DILUTIONS OF 20158 ANTISERUM												TYPE OF AGG.	TREATMENT OF TEST
			40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960	Control		
A	Nor.	37	4	4	4	4	4	4	4	4	3	2	1	—	H	
		55	3	3	3	3	3	3	3	3	2	1	±	—	T	B. S.
			2	1	1	±	±	—								A. S.
B	100 C	37	2	2	2	2	1	1	±	—					O	
		55	4	4	4	4	4	4	4	4	3	1	—		O	B. S.
			3	3	3	3	3	3	3	3	2	±	—		O	A. S.
C	Nor.	37	4	4	4	4	4	4	4	4	3	2	1	—	H	
		55	4	4	4	4	4	4	4	4	3	1	—		O	B. S.
			3	3	3	3	3	3	3	3	2	1	—		O	A. S.
D	Nor.	37	4	4	4	4	4	4	4	3	2	1	±	—	H	
		55	4	4	4	4	4	4	4	3	2	1	—		O	B. S.
			3	3	3	3	3	3	3	2	1	±	—		O	A. S.

A = normal 20158 antigen in 20158 antiserum (control).

B = heated 20158 antigen in 20158 antiserum to restore O agglutination.

C = antiserum 20158 diluted in saline or alcohol extracts of culture 20158.

D = antiserum 20158 adsorbed with culture 14011, which gave T agglutination in 14011 antiserum but did not agglutinate in 20158 antiserum.

4, 3, 2, 1, ±, — = degrees of agglutination from complete to negative.

B. S. and A. S. = before shaking and after shaking, respectively.

Vi-positive cultures to 99 C or higher dissociated the Vi antigen from the cell but the antigen in question was not destroyed. In the present work heavy saline suspensions of *P. intermedium*, 20158, which gave excellent T agglutination, were heated to 99 C and higher for 2.5 hours. After centrifugation the supernatant fluid was removed and the packed cells were washed in saline. The heated washed cells yielded only O agglutination in 20158 antiserum (table 1, B). To determine the effect of the supernatant fluid of heated 20158 upon T agglutination the following tests were set up. Antiserum 20158 was diluted serially from 40 to 40,960 in the supernatant fluid. The same dilutions made

with the antiserum in saline served as a control. To both sets of dilutions saline suspensions of antigen 20158 were added. After 2 hours at 37 C both tests showed H reactions; after 18 hours at 55 C the tests in saline (control) showed T, whereas the tests in the supernatant showed only O agglutination (table 1, C). It was evident that some thermostable substance in the supernatant fluid of heated 20158 culture inhibited T reactions and restored O agglutination. Heated and unheated alcohol-soluble, saline-soluble extracts of culture 20158 (for details of preparation see Stuart and Kennedy, 1948) also showed inhibition of T agglutination.

Saline and alcohol extracts of one serotype of *P. intermedium* inhibited T agglutination and restored O agglutination to many other serotypes of this group of organisms. Moreover, extracts of most *P. intermedium* cultures showing T reactions inhibited T agglutination in *S. alkalescens, Escherichia coli*, type 29911 (Stuart, Wheeler, and McGann, 1946), and other cultures showing T reactions. It was evident, therefore, that the substance that inhibited T agglutination was common to cultures of the family *Enterobacteriaceae* that were otherwise antigenically unrelated.

All saline and some alcohol extracts completely inhibited T reactions as shown in table 1, C. Most alcohol extracts restored perfect O agglutination in the lower dilutions of the antiserum, followed by a transitional type of agglutination in the intermediate dilutions, with perfect T reactions in the higher dilutions. This type of reaction was recorded as "O-T" (granular to thread).

Adsorption of 20158 antiserum by a few serologically unrelated organisms that gave T reaction completely restored O agglutination to the adsorbed antiserum (table 1, D). Adsorption by other organisms that gave T agglutination resulted in an O-T type of reaction.

Since Williams, Bloor, and Sandholzer (1939) found that phospholipids constituted a major portion of the lipid extracted from bacteria by alcohol, a 2 per cent suspension of lecithin was used to investigate the inhibition of T agglutination. Although not so efficient as alcohol extracts of *P. intermedium*, lecithin showed marked inhibition of T agglutination but always gave an O-T reaction.

T agglutination in *P. intermedium* cultures was frequently associated with the opaque and translucent colony variation. Cultures from opaque colonies of any one strain gave only T agglutination, but cultures from translucent colonies of the same strain gave characteristic O reactions.

Living and heated suspensions of cultures exhibiting T agglutination failed to react in antiserums prepared from two cultures possessing alpha antigen or in antiserums against several cultures containing Vi antigen. (The alpha antiserums were prepared by Dr. K. M. Wheeler, deceased, Connecticut State Department of Health, Bureau of Laboratories, Hartford, Connecticut.) However, alcoholic extracts of cultures exhibiting T agglutination fixed complement in the presence of antiserums made from alcoholic extracts of Vi-positive cultures. The relationships established by alcoholic extracts of the cultures in question and antiserums prepared from such extracts are obscure and confusing and should be studied further. Possible relationships between cultures showing T agglutination and the K antigens of Kauffmann (1947) were not investigated.

DISCUSSION

The T type of agglutination encountered in this work definitely seems to be another form of so-called "O-inagglutinability." There is probably more than one antigen or substance concerned in T agglutination, but detailed studies on this point were not undertaken. Furthermore the term T agglutination is one of convenience and no taxonomic recommendation is implied.

The antigen initiating T agglutination belongs to the general group of antigens that confer some form of O-inagglutinability upon cultures containing them. This antigen is found in many *P. intermedium* cultures and occasionally in other members of the family *Enterobacteriaceae*. In this category belong the Vi antigen of Felix and Pitt (1934), the alpha antigen of Stamp and Stone (1944), the K antigens of Kauffmann (1947), and the T antigen. One or another of these antigens is found in strongly encapsulated cultures, in other cultures that are weakly encapsulated, and in still other cultures that do not appear to have capsules. When cultures are heated, O-agglutinability is restored to those possessing the above-named antigens, but generally the temperature required to restore O agglutination increases with the degree of encapsulation. In certain instances all of the specified antigens, particularly the Vi, are associated with the raised opaque, rather than the flat translucent, colonial form.

Judging from studies in this laboratory of the serological relationships of thousands of cultures in the family *Enterobacteriaceae*, the number of antigens conferring some form of O-inagglutinability could be expanded rapidly if it seemed desirable. This group of antigens is important in studying serological relationships and in diagnostic work because they tend to obscure homologous and heterologous somatic reactions. The use of heated antigens will avoid most of the difficulty, but it must be remembered the degree of heat required to produce the best results is not the same for all these antigens.

CONCLUSIONS

Thread agglutination is a manifestation of O-inagglutinability. Characteristic O agglutination is restored by heating antigens that give T reactions. The substance responsible for T agglutination is thermostable, easily dissociated from the cell, and seems to be associated with a phospholipid constituent of the cell.

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THE EFFECTS OF NITROGEN MUSTARD ON ESCHERICHIA COLI[1]

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A review of the genetic literature on chemically induced mutations would include many references extending back for over thirty years to the pioneer studies of T. H. Morgan (1914). The earlier studies were either negative or inconclusive. Unequivocal demonstration of a powerful chemical mutagen was not made until a group of geneticists working in England during the recent war began investigating the effects of mustard gas (Auerbach *et al.*, 1947). By means of the CLB test Auerbach and her colleagues were able to show that sex-linked lethals in *Drosophila* were induced with a frequency comparable to that previously attained only with short-wave high energy radiation. Subsequently nitrogen and sulfur mustards were successfully employed to induce genetic changes, these compounds having the general formulae: $O(CH_2CH_2 \cdot S \cdot CH_2CH_2Cl)_2$; $CH_3N(CH_2 \cdot CH_2Cl)_2$; and $N(CH_2 \cdot CH_2Cl)_3$. Their biochemical properties are thought to depend on intramolecular cyclization to form onium cations of high reactivity and possessing the ability to alkylate many cellular constituents. Possibly the alkylation of nucleoprotein through carboxyl or amino linkages is attained. The reactivity of enzymes, particularly phosphokinases, with mustard compounds and the inhibition of oxidative and glycolytic mechanisms by ethylenimonium and sulfonium cations emerge as well-established biochemical properties. A single site of action or specific substrate in all probability does not exist for these highly reactive substances, and geneticists are compelled in the present state of limited knowledge to use the mustards empirically as chemicals capable of producing a large number of diverse biochemical changes in the cell. Among these changes we may include mutations in microorganisms (Horowitz *et al.*, 1946; Tatum, 1947).

Historical retrospection in the field of induced mutation reveals that a suitable method for the demonstration of mutants is at least as important as choice of an appropriate mutagenic agent. Muller's contribution of the CLB technique in *Drosophila* and more recent analogous techniques form the keystone to all studies of mutation frequency in this organism. Controlled studies of mutation frequency in bacteria by measuring the rate of change to bacteriophage resistance represent use of but one of a variety of criteria available to the bacteriologist interested in genetic modifications (Luria, 1947). Phage resistance affords a method at least as simple as the use of altered enzyme specificities or biochemical growth requirements to measure mutation frequency.

In addition, the work of Demerec and Latarjet (1946) with X-rays and ultraviolet light as mutagenic modifiers of mutation rate to phage resistance provides

an interesting parallel to related studies with chemicals. Therefore we have chosen the effect of nitrogen mustard on mutation to phage resistance in *Escherichia coli* as the subject for investigation.

MATERIALS AND METHODS

The strains of bacteriophage employed were T1 and T2, in the nomenclature of Demerec and Fano (1945). Strain T2 served in testing for contaminants by the method that these authors have described. Fourteen stocks of radiation-resistant *E. coli* (B/r) were obtained from Dr. E. Witkin. Of independent origin, the B/r strains were known to be identical in their degree of resistance to X-rays and to ultraviolet light (Witkin, 1947a). A normal nonmotile strain of *E. coli*, designated as B, was the stock from which all cultures were originally derived.

The method employed in testing for induced mutation by chemical agents consists of two essential components: (1) detection of any change in the relative proportion of phage-sensitive to phage-resistant cells after exposure to a chemical, and (2) test to determine whether any shift in the relative proportions of phage-sensitive cells is due to differential susceptibility of resistant and sensitive individuals to the chemical agent under analysis as a potential mutagen. The actual procedure utilized in detecting alterations of relative proportion in sensitive cells (B) to cells resistant to the phage T1 involves initially the preparation of a culture containing low numbers of the resistant mutants. Since resistance is to phage T1, resistant cells are conventionally described as B/1. For simplification, mutants described in this investigation as B/1 include two classifications: cells resistant to T1 only (B/1), and cells becoming simultaneously resistant to T1 and T5 (B/1, 5). The preparation of stocks proceeds as follows: One-tenth ml of a 10^{-6} dilution obtained from a 24-hour, aerated, low background nutrient broth culture is used to inoculate 8 to 10 flasks containing 150 ml of medium. The inoculum will contain approximately 2×10^2 sensitive organisms, with a low probability that any resistant cell is carried in one inoculation. After forced aeration at 37 C for 24 hours the series of inoculated flasks will be found by dilution assays to contain approximately 2×10^9 bacteria per ml. By the plating of undiluted 0.1-ml aliquots from each culture on nutrient agar previously spread with 0.1 ml of undiluted phage T1 all sensitive cells are lysed. Thus only B/1 cells remain to form colonies on the agar surface. The proportion of B/1 to B is obtained by comparing for each individual culture the number of B/1 colonies obtained with the titer as determined by dilution assay on unphaged plates. This proportion is defined as the background. For example, if a culture contains 3.5×10^9 bacteria per ml and 35 colonies appear from a 0.1-ml sample plated in the presence of T1, we may say that the background is 10 B/1 per 10^6.

An obvious aid in studies of mutation is a low number of mutants spontaneously present in the culture to be tested. A low background number of less than 20 B/1 per 10^8 is obtained only when mutation of B to B/1 occurs rarely in the growth of a culture and late enough in the growth phase so that descend-

ants of the mutated cells do not eventually constitute a large proportion of the saturated population. In an actively growing broth culture spontaneous mutation of B to B/1 occurs with a frequency in the order of 1 per 10^8. The occasional appearance of spontaneous mutants in the early growth of cultures inevitably provides a proportion of high background preparations that are discarded as unsuited for the desired experimental purpose.

Low background cultures are centrifuged, and the sedimented cells from several independent broth preparations are pooled to provide at least 3 ml of cells. Five-tenths ml of the sedimented cells are added to an equal volume of saline and 1 ml of broth, giving a total volume of 2 ml with a titer of 2 to 5 × 10^{10} per ml. This sample is assayed to determine exact titer and background. The addition of saline and broth assumes a meaning when we compare the assay titer and background with the remaining aliquot of cells obtained by the original centrifugation and set aside for experimentation. This remaining aliquot undergoes a dilution corresponding to the sample employed for determination of titer and background. The first dilution occurs when an equal volume of buffered nitrogen mustard is added to the cell suspension. The material is then incubated at 37 C for 1 hour. A later dilution takes place at the termination of chemical treatment by the addition of a quantity of chilled nutrient broth equal to the combined volume of cells and saline. In each aliquot the cellular suspension thus constitutes 25 per cent of the total liquid volume. Originally, addition of broth to the experimental preparation was intended to retard the reaction between cells and chemical by dilution and by reduction of the temperature from the experimental level of 37 C. Later it was found that, when cells have been incubated at 37 C with 0.1 per cent nitrogen mustard for 1 hour, the reaction between chemical and substrate has proceeded at a decelerated rate to a point where further exposure produces insignificant change in survival.

Determination of zero-point mutations. Following treatment it is necessary to determine the extent of killing. Routine assay tubes are set up at dilutions of 10^{-2}, 10^{-4}, and 10^{-6}, providing an index of survival by subsequent plating. One-tenth ml of the chemically treated cells is then placed on each of 20 nutrient agar plates that have been previously prepared by the surface spreading of 0.1 ml of undiluted T1 phage. After incubation of the plates intended for assay it is possible to determine the number of viable cells per ml following chemical treatment. The quantity of viable cells plated on 20 phaged nutrient agar plates must correspond to twice the assay number (0.1 ml × 20). The number of B/1 cells is determined directly by counting the colonies on phaged plates following their incubation, permitting the calculation of B/1 per 10^8 survivors. A comparison may then be made of the proportion of B/1 per 10^8 cells before and after chemical treatment. Simple subtraction of the background number from the final relative number of resistant cells provides a direct index of mutation induced in the nondividing cells during chemical exposure, providing selection is ruled out by the necessary experiments to be conducted concurrently. For example, if a stock containing 3 B/1 cells per 10^8 as background is treated by a chemical known to be nonselective, with a final proportion of 250 B/1 per 10^8

chemically treated viable organisms, the number of induced mutants is 250 minus 3, or 247 B/1 per 10^8 cells. In experiments with radiation Demerec and Fano (1945) described the mutations that occur in nondividing cells as zero-point mutations. Refrigeration of all cells at 12 C for at least 12 hours prior to experimental use ensures the absence of cell division during 1-hour exposure to the chemical and places mutations attained by this technique in the category of zero-point mutants.

Determination of delayed mutations. By a supplementary method the class of B/1 known as delayed mutations may be obtained. Such mutations appear in the descendants of treated cells with a frequency greatly exceeding the spontaneous rate of occurrence. They are thought to result from labile modifications of genetic structure requiring the intervention of one or more cell divisions before expression. Delayed mutations are obtained by allowing treated cells to multiply on *unphaged* plates for sufficient time to extend beyond the stationary phase (4 to 6 hours). Assay for total cell number is then made by washing cells carefully from the agar surfaces, with dilution at varied levels to provide an estimate of total cell number attained by incubation from the original treated 0.1 ml spread on the plate. Another series of plates is phaged by exposure to T1, aerosolized from a commercial nebulizer (DeVilbiss no. 40). Aerosol avoids disturbance of bacterial colonies which must remain *in situ* for accurate determination of delayed B/1 mutants. Each colony that appears after delayed phaging represents either a zero-point mutation or a mutant that appears during the reproduction of sensitive cells through division. If the number of zero-point mutants has been determined in another series of plates, the delayed mutants may be derived by comparing the number of additional mutants with new cells arising during the incubation period and assayed by unphaged controls (Demerec and Latarjet).

One advantage of the technique for determining delayed mutations is that selection cannot play a role in the experimental result. All delayed mutants must have arisen *de novo* since they represent a number larger than the total number of phage-resistant cells (B/1) available for selection to act upon, as revealed by the total number of colonies present on plates phaged without prior incubation.

Activity of methyl-bis (beta-chloroethyl)amine hydrochloride as related to pH. It is known that aqueous solutions of the salts of nitrogen mustards are acid and relatively stable. At physiological hydrion concentrations the solution becomes highly reactive with rapid union of the ethylenimonium cation and available anionic groups. It has been a routine procedure to buffer the chemical prior to the addition of bacteria. Reaction between nitrogen mustard and buffer will rapidly utilize available ethylenimonium cations, as will reaction with the solvent. Consequently, the buffering of an aqueous solution of methyl-bis(beta-chloroethyl)amine hydrochloride, or HN-2, by Na_2HPO_4 results in a very rapid alteration in the toxicity of the solution. Since any test for mutagenesis requires some measure of control over toxicity of the mutagenic agent, an investigation of the effect of Na_2HPO_4 on HN-2 assumes significance. Addition of 8 ml of

M/4 Na$_2$HPO$_4$ to 2 ml of 1 per cent HN-2 results in a rise of pH from 4.5 to 6.8. Addition of equal volumes of this freshly prepared 0.2 per cent buffered HN-2 to a cell suspension with subsequent incubation at 37 C for 1 hour gives a survival rate of 1.3 per 10^8. If the buffered HN-2 is not added to another sample of cells until 5 minutes after the addition of buffer, the survival value becomes 1 X 10^5 per 10^8. The reduced physiological activity of buffered HN-2 dependent upon elapsed time before the addition of cells is represented graphically in figure 1.

It is apparent that the toxic effects of HN-2 in buffered solution decrease at a decelerating rate with time and that after 15 minutes' contact with buffer only a minor residual toxicity remains. The earlier experiments conducted in this study were made without knowledge of this fact, accounting in part for variations in the degree of killing obtained by identical concentrations of buffered HN-2.
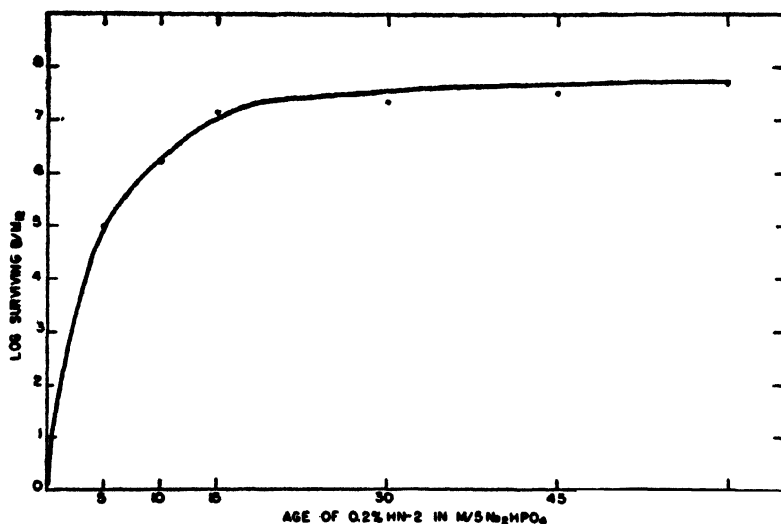
Figure 1. Effect of aging HN-2 in buffer before addition to *E. coli.*

INDUCTION OF HN-2 RESISTANCE IN E. COLI STRAIN B

Preliminary experiments established the fact that no unequivocal mutagenic effect of HN-2 on strain B is demonstrable at concentrations of 0.005 or 0.04 per cent with 1-hour exposure at 37 C. At the higher concentration survival was reduced to 0.04 per cent, yielding only two colonies in 20 plates (vol. 2 ml of treated cell suspension). The obvious method of attempting to increase the proportion of delayed mutants (eliminating selection)' by increased concentration or time of exposure was made impractical by the extreme toxicity of the compound. Since strains of bacteria resistant to toxic agents may be obtained by the selection of mutant cells, the possibility arises that the production of a strain of *E. coli* resistant to HN-2 would allow higher concentration of the chemical to be employed in tests for mutagenesis. Several strains of mustard-resistant *E. coli* were produced by seeding ca. 2 X 10^9 cells in petri plates, which were then

filled with 10 ml of proteose no. 3 agar at HN-2 concentrations ranging from 0.1 to 0.4 per cent. One colony from the plate having the lowest number of survivors was picked to provide a new strain of cells. Strains have been designated as B/M1, B/M2, ... B/M12, the numerical notation indicating the number of exposures to HN-2 preceding the isolation of a particular mustard-resistant stock. Thus strain B/M12 was derived from B/M11. Increase in resistance to HN-2 was found to occur progressively and is not an all-or-none phenomenon. A comparison of different strains of *E. coli* exposed to HN-2 is presented in table 1.

TABLE 1

Resistance of E. coli strains to HN-2

STRAIN	SEEDING	COLONIES AT VARIED CONCENTRATIONS OF HN-2							
		0.35	0.3	0.25	0.12	0.15	0.1	0.05	0.025%
	Bact. per 0.5 ml								
B	2×10^9	—	—	2	41	557	$>10^4$	$>10^4$	$>10^4$
B/M1	3×10^9	—	1	10	4,100	$>10^4$	$>10^4$	—	—
B/M2	4×10^9	—	30	215	$>10^4$	$>10^4$	$>10^4$	—	—
B/M7	1×10^9	202	4,500	$>10^4$	$>10^4$	—	—	—	—
B/r*	1×10^9	22	1,500	$>10^4$	$>10^4$	—	—	—	—

* Radiation resistant.

TABLE 2

Induction of phage-resistant mutants of B/M12 by 0.1 per cent HN-2, aged three, two, and one minute in M/5 Na₂HPO₄ before addition to cells

PER CENT SURVIVAL	AGE OF BUFFERED HN-2	SEEDING	ZERO-POINT MUTANTS
	min		
0.9	3	9.2×10^7	1.1×10^1 per 10^8
0.007	2	4.1×10^5	2.4×10^2 per 10^8
0.0004	1	1.5×10^4	2.3×10^3 per 10^8

It is apparent that strains exposed to HN-2 should provide through selective mechanisms a form suitable for further studies of mutagenesis, permitting higher concentrations of HN-2 to be used. Although a high degree of resistance was attained in strain B/M7, transfers and selection of surviving colonies were continued to derive strain B/M12, which was used for further study. HN-2 was at the same time found to be relatively nontoxic to a radiation-resistant strain (B/r), an observation extended by additional experimentation (Bryson, 1947).

Mutagenic effects of HN-2 on B/M12. A 0.1 per cent solution of HN-2 in M/5 Na₂HPO₄ was selected as the optimum concentration for tests of induced phage resistance by mutation of B/M12 to B/M12/1. The toxicity of HN-2 was varied by allowing different intervals of time to elapse before cells were added to the buffered chemical. A summary of three experiments is given in table 2.

Experiments were conducted by seeding 20 phaged plates, each with 0.1 ml

of HN-2-treated cells. Chemical treatment was for 1 hour at 37 C. It is observed that the proportion of zero-point mutants increases with decreased survival. To what extent this is a real and not an apparent induction of mutation depends on the relative sensitivity of B/M12 and B/M12/1 to HN-2. Supplementary experiments show that the phage-sensitive stock is more resistant to HN-2, eliminating the possibility of selection in the interpretation of data presented in table 2. Delayed mutation where selection is not a factor gave 370 delayed mutants per 10^8 at 0.007 per cent survival with 8.3 cell divisions, and 440 per 10^8 at 0.9 per cent survival with 2.2 cell divisions. The rate of induced and spontaneous mutation falls off in cell divisions following a stationary phase, being highest in the first division.

Resistance of B/M12 to ultraviolet light. The effect of nitrogen mustard on cells and tissues has often been compared to that of short-wave radiation. An interesting question therefore arises: Is strain B/M12 more resistant to radiation than the normal B cells? The question was investigated by exposing cells spread over the surface of nutrient agar plates to the emanations of a G. E. mercury vapor arc lamp. There can be no doubt that HN-2-resistant strains are also resistant to the lethal effects of ultraviolet light. Five of the numerous ultraviolet-resistant strains provided through the courtesy of Dr. E. Witkin were tested simultaneously. There appears to be no significant difference between HN-2 and radiation-resistant stocks (B/r nos. 34, 14, 23, 32, and 37). Simultaneous protection against the toxic properties of both ultraviolet and nitrogen mustard through mutation suggests a common biochemical effect of these two agents. The similarity in resistance is shown graphically in figure 2. Survivors per 10^8 cells are indicated by the ordinate. The common pattern of resistance has been discovered independently by the Italian geneticist, L. Cavalli (1948).

Reciprocal relationship of resistance to HN-2 and radiation. The demonstration that strains of *E. coli* resistant to HN-2 also resist the toxic effects of ultraviolet light suggests that the protective mechanism may be identical. Theoretically four alternative situations might exist: HN-2 resistance and radiation resistance; HN-2 resistance and radiation sensitivity; HN-2 sensitivity and radiation resistance; and sensitivity to both agents. If HN-2 resistance and radiation resistance are identical or coupled, only two states may prevail, either resistance or sensitivity *in toto.* As a test for the four alternative states, nutrient agar plates were spread with approximately 10^6 cells of eight representative bacterial strains. One-half-inch disks of filter paper were placed on each side of the petri plates after saturation with 0.03 ml of 0.25 per cent HN-2. One side of each plate, including a disk, was then shielded while the remaining surface was exposed to 50 ergs/mm²/second of ultraviolet light. After 5 hours of incubation at 37 C the exposed half-plate was again irradiated at 1,800 ergs (double irradiation technique of Witkin). The diagram shown in figure 3 gives the four possible consequences of experimental treatment. Crosshatched areas indicate the portion of plate shielded from radiation. As shown in figure 3, only two categories of plates were found. Cells resistant to HN-2 as revealed by the growth

of cells in contiguity with the filter paper disks were also resistant to radiation, allowing survival on the unshielded portion of the plate. Thus all tested strains were included in two categories: (1) sensitive to both HN-2 and ultraviolet, or
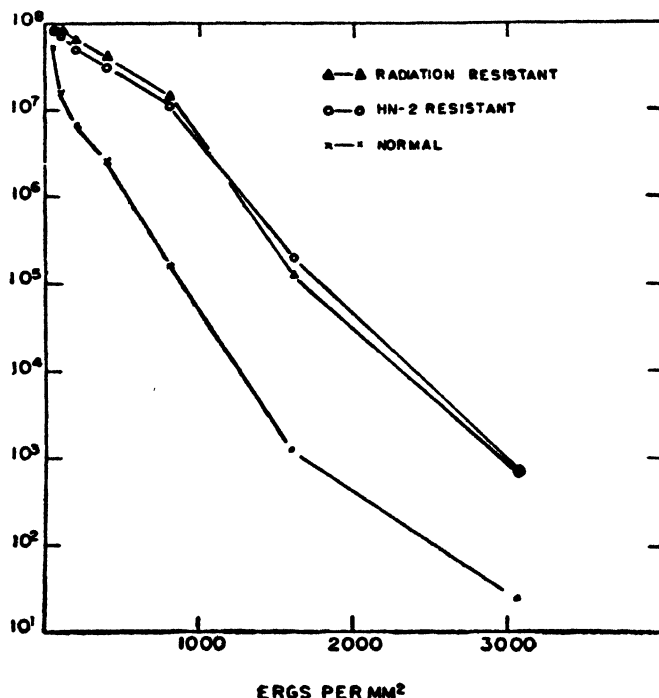
Figure 2. Relative resistance of normal, radiation-resistant, and mustard-resistant strains of *E. coli* to ultraviolet light.
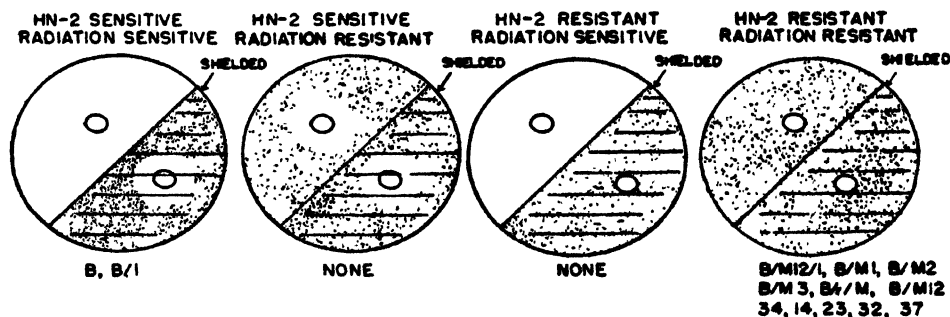
Figure 3. Distribution of *E. coli* strains exposed to methyl-bis(beta-chloroethyl)amine hydrochloride and ultraviolet radiation.

(2) resistant to both agents. The resistant strains consisted of three independent radiation cultures (B/r) and three cultures that had been exposed to the effects of HN-2 in previous experiments.

One of the HN-2-resistant strains was also phage-resistant—B/M12/1. Zones of inhibition surrounding the paper disks averaged 5 mm in width, measured radially from the disk border. A few resistant colonies could be found in HN-2- and ultraviolet-inhibited zones, but in insufficient quantity to affect the validity of the test.

As a further procedure, representative strains were inoculated from slants into nutrient broth and aerated at 37 C for 24 hours. Cultures were then added to petri dishes at a dilution of 10^{-6} in 0.5-ml quantity. Exactly 10 ml of proteose no. 3 agar containing varied concentrations of HN-2 were then mixed in
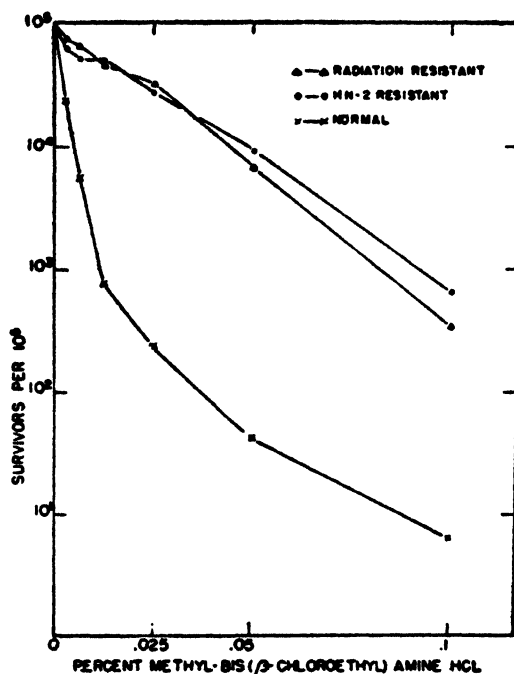
Figure 4. Relative resistance of normal, radiation-resistant, and HN-2-resistant strains of *E. coli* to methyl-bis(beta-chloroethyl)amine hydrochloride.

each inoculated plate with the bacterial suspension and incubated. Control plates were inoculated simultaneously, yielding an average number of 680 bacteria per plate. Thirteen independent radiation-resistant stocks showed 2 to 77 survivors at a concentration of 0.1 per cent HN-2. Stock B/M12 yielded 7 survivors (colonies). No cells of strain B survived a concentration of 0.025 per cent HN-2.

It is concluded that all 14 radiation-resistant strains are able to grow in concentrations of HN-2 that inhibit the growth of the normal B strain. If a larger quantity of normal cells are used, some survivors may be found in plates containing 0.025 per cent HN-2 (30 survivors in 94,000).

To perform an experiment analogous to the ultraviolet exposure data pre-

sented in figure 2 it was necessary to determine the required inoculum size to yield a countable plate after treatment of cells with high concentrations of HN-2 in proteose no. 3 agar.   After considerable experimentation, killing limits were determined for varied concentrations and the resulting data are given in figure 4. Because HN-2 loses its toxicity with time after addition to proteose no. 3, the experiment was duplicated reversing the order in which HN-2-containing media were added to the different strains of cells.   Points on the curves represent the average of six HN-2-resistant stocks compared with six radiation-resistant stocks. Strain B and B/1 served as a control, their values before averaging being less than the mean survival of the HN-2- and radiation-resistant strains.   Inspection of figure 4 amply justifies the conclusion that radiation-resistant *E. coli* closely resembles HN-2-resistant cells in relative insensitivity to toxic effects of the chemical.

DISCUSSION

The conclusion may be drawn that although mutation of B to B/1 is not certainly induced by HN-2, the conversion is readily accomplished in a strain resistant to the chemical; in the presence of methyl-bis(beta-chloroethyl)amine hydrochloride, B/M12 cells may be changed to B/M12/1.   Previous reports have indicated that HN-2 has the capacity to induce mutation (Auerbach *et al.*, 1947).   The studies of Dr. E. Witkin (1947*b*) indicate that the variety of chemicals capable of inducing mutation is greater than had been anticipated.   No practical advantages attend these findings at the present time, since the mutagenic influence of chemicals may more easily be duplicated with X-rays or ultraviolet radiation.   Continued research with microorganisms may eventually uncover differences in the types or relative frequencies of genetic changes depending on the mode of induction, as previously observed by geneticists comparing the effects of radiation of varied wave length.   A continued search in the field of chemically induced mutation offers the best hope for directed mutation, analogous to the transmutation effect of desoxyribonuclease on pneumococci as described by Avery, MacLeod, and McCarty (1944).   Compared with the random action of ionizing radiations, specific chemicals should offer a more precise mechanism for altering the chemical structure of the cell to produce directed genetic modification.   Highly reactive chemicals, including HN-2, would be expected to offer the least specificity.

It is believed that the most interesting result of this study is the finding that resistance to radiation and to methyl-bis(beta-chloroethyl)amine hydrochloride may involve an identical mechanism.   A number of interesting hypotheses may be advanced to account for protection against these agents by a single genetically determined biochemical change.   However, there exist no biochemical facts on which a sound hypothesis might be based.   Until such facts are available, it therefore appears unduly speculative to indulge in interpretations of the mechanism of resistance.   Present efforts should be directed rather to the analysis of enzyme activity, metabolic processes, and polymerization characteristics of the resistant cell structure.

SUMMARY

Methyl-bis(beta-chloroethyl)amine hydrochloride (HN-2) is capable of inducing mutations to phage resistance in a strain of *Escherichia coli* specifically resistant to the toxic effects of this chemical.

The resistance of selected strains of *E. coli* to the lethal effects of HN-2 is identical or related to ultraviolet radiation resistance. Conversely, strains of *E. coli* that have mutated to ultraviolet resistance are simultaneously relatively insensitive to killing by HN-2.

Resistance to HN-2 and ultraviolet light therefore appears to result from related genetic changes and suggests a similarity in the mode of action of these two agents on the bacterial cell.

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CHARACTERISTICS OF TARTRATE-FERMENTING SPECIES OF CLOSTRIDIUM

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While establishing methods for the control of spoilage in the tartrate recovery plants of California, Vaughn *et al.* (1943a,b; 1946) isolated a number of D-tartrate-decomposing microorganisms, including several mesophilic clostridia. These organisms, among the most active fermenters of D-tartrate, are of historical as well as economic interest, for it was in a tartrate medium that Pasteur (1863) encountered similar organisms in his study of anaerobiosis. This investigation was undertaken to determine the characteristics of a number of these D-tartrate-fermenting clostridia, for, despite the early observation of anaerobic tartrate decomposition, to the writers' knowledge a pure culture study of the tartrate-fermenting species of the genus *Clostridium* has never been reported.[1]

EXPERIMENTAL RESULTS

Isolations were made from 18 different sources. The majority of cultures were obtained from spoiled crude D-tartrate or tartrate recovery equipment.[2] Several strains were isolated from vineyard soil. One isolate was obtained from marine mud. Fifteen of the cultures were obtained by direct isolation from the source material placed on glucose agar. The eight remaining strains were obtained by an enrichment technique involving D-tartrate media. Fifty-four additional cultures representing twenty-five species of the genus *Clostridium* were used as controls. For the majority of the cultures in this latter collection the authors are indebted to Dr. Elizabeth McCoy, Dr. C. E. Clifton, Dr. L. S. McClung, Dr. F. W. Tanner, Dr. Sanford S. Elberg, Dr. K. W. Raper, and Dr. E. J. Cameron. The remaining control cultures were obtained from the American Type Culture Collection. Conventional techniques were used throughout the course of the study. References rather than detailed descriptions will serve to acquaint the reader with them.

Characteristics of the Bacteria

A majority of the cultures possessed similar morphological characteristics. They were large, motile rods with rounded ends, occurring singly, in pairs, in chains, and occasionally in long filaments. The cells were gram-positive in very young cultures, but became gram-negative from 5 to 10 hours after turbidity appeared in liver infusion broth. The cultures all gave typical granulose reactions with iodine.

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[1] For a summary of the early literature concerning the microbial decomposition of tartrates, see Vaughn, Marsh, *et al.* (1943b, 1946).

[2] For an understanding of the tartrate recovery processes consult Marsh (1943).

The cultures varied in their ability to produce spores; some sporulated profusely, others sparingly. The spores were always elliptical and generally contained in a subterminal position inside the sporangium. Sporulation resulted in distinct swelling of the cells.

Typical colonies grown on tryptone glucose agar (3 days at 37 C) averaged 1.5 mm in diameter and were smooth, white, opaque, convex, glistening, and circular with irregular margins. On tryptone ammonium D-tartrate agar the colonies were minute and appeared translucent or transparent.

The differential characteristics of the 23 tartrate-fermenting isolates are shown in table 1. None of the cultures softened or digested coagulated egg albumen, blackened or digested brain medium, or liquefied gelatin. The bacteria did

TABLE 1

Characteristics of the tartrate-fermenting isolates

NUMBER OF CULTURES	DECOMPOSITION OF PROTEINACEOUS MATERIALS			FERMENTATION AS INDICATED BY GAS PRODUCTION AND CHANGE IN pH											TENTATIVE SPECIES ALLOCATION (AFTER SPRAY, 1948)
	Coagulated egg albumen	Brain medium	Gelatin liquefaction	D-Xylose	Glucose	Lactose	Maltose	Sucrose	Galactose	Glycerol	Mannitol	Salicin	Inulin	Starch*	
	Number of cultures showing positive reactions at 37 C														
12	0	0	0	12	12	12	12	12	12	10	12	12	7	12	*Clostridium butyricum*
8	0	0	0	8	8	8	8	8	8	8	8	8	2	0	*Clostridium beijerinckii*
1	0	0	0	1	1	1	1	1	1	0	0	1	0	0	*Clostridium pasteurianum* (Strain N)
1	0	0	0	1	1	0	1	1	0	0	0'	0	0	0	? (Strain Q)
1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	? (Strain R)

* Soluble and insoluble starches were tested. Demonstration of extracellular amylase as well as fermentation with gas production and change in pH were criteria for decomposition.

not produce indole in tryptone medium even when galactose, D-xylose, or ammonium D-tartrate were substituted for glucose (Spray, 1936; Evans *et al.*, 1942). Hydrogen sulfide was not produced from the sulfur-containing constituents of tryptone. The organisms did not grow in tryptone broth in the absence of a fermentable substrate. Strain Q was the only isolate that reduced nitrate to nitrite.

The cultures all grew well at temperatures ranging from 30 to 37 C. They grew slowly at 25 C but did not grow at all at 40 C (temperature of the medium).

The minimum pH value for the growth of these bacteria in tartrate media was 4.7. The majority of the strains, however, could not grow at pH values below 5.1. The desired pH values of the tartrate media were obtained as described by Vaughn *et al.* (1946). The minimum pH value for growth was determined

by an adaptive transfer technique (Gililland and Vaughn, 1943). It was not determined whether the effect on growth was caused by the hydrogen ion activity or was in part the result of the toxicity of undissociated tartaric acid.

The D-tartrate-fermenting cultures are representative of the saccharolytic clostridia. The majority (12 isolates) closely resemble the type species *Clostridium butyricum* regardless of their ability to ferment glycerol.[*] The eight strains allocated to the species *Clostridium beijerinckii* possess the general characteristics of *Clostridium butyricum* but are separated from it by their inability to ferment starch.

The three remaining cultures, N, Q, and R, differ markedly from the other isolates studied. They are more difficult to grow and grow more slowly. Strain N has been allocated tentatively to the species *Clostridium pasteurianum* despite the fact that it ferments lactose. It will be noted that strain R ferments glucose and mannitol, whereas strain Q ferments D-xylose, glucose, maltose, and sucrose, although they both attack D-tartrate. On morphological grounds strain R is similar to the other identified isolates. Strain Q, however, characteristically forms surface colonies that are flat and circular, have myceloid edges, and are dull yellow in color. Because of these differences it is felt that specific designations for these two isolates are not warranted until further investigations have been made.

Some supporting evidence to strengthen the specific allocations for the majority of the strains (N, Q, and R excepted) was obtained by determination of the end products of unbuffered glucose fermentations by three representative isolates. The chief end products of the glucose fermentations by these three cultures were carbon dioxide, hydrogen, and butyric and acetic acids. The molar ratio of butyric to acetic acid was approximately 4 to 1.

The production of only very small amounts of isopropyl alcohol by one strain, and only trace quantities of neutral volatile products by the other two cultures, plus the absence of acetoin and 2,3-butanediol from the fermentation products of all three isolates, is considered additional evidence for placing the D-tartrate-fermenting cultures in the *Clostridium butyricum* group. (See McCoy *et al.*, 1930; Langlykke *et al.*, 1935; Osburn *et al.*, 1937.)

End Products of D-Tartrate Fermentation

Since earlier workers apparently had not used pure cultures in their studies of the anaerobic fermentation of tartrate, it was desirable to determine the end products formed from tartrate fermentations by several of the pure cultures just described. Four representative cultures (N, Q, and R excepted) were used. They were grown in a medium containing 0.2 per cent yeast extract (Difco), 0.1 per cent tryptone (Difco), 0.1 per cent dibasic potassium phosphate, and traces of ferrous, manganous, and magnesium salts, to which were added concentrations of ammonium D-tartrate consisting of 1.0, 1.5, 2.0, and 4.0 per cent,

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[*] Species allocation follows Spray (1948) in *Bergey's Manual of Determinative Bacteriology*. Some authorities consider that glycerol fermentation is variable among the strains of *Clostridium butyricum*. See Spray (1936).

respectively. The reactions of the media containing the various concentrations of D-tartrate were between pH 6.8 and 7.0 after sterilization. The inoculated spores germinated and grew readily in the cultures containing from 1.0 to 2.0 per cent ammonium D-tartrate with complete utilization (96 per cent ± 5 per cent) of the tartrate in all cases within 12 to 18 hours after the first visible signs of growth. After 3 weeks' incubation at 30 C, only one of the four cultures tested grew in the medium containing 4.0 per cent ammonium D-tartrate. The final pH value of the fermented medium varied from 7.5 to 8.5.

The major end products formed from D-tartrate by these bacteria were carbon dioxide, hydrogen, and acetic acid. Butyric acid was formed but in much smaller amounts than acetic acid. The molar ratio of acetic to butyric acid was approximately 10 to 1. The fact that acetic acid is the principal acid formed in the tartrate fermentation, rather than butyric acid as in the fermentation of glucose, can be explained by the more oxidized state of tartrate (Johnson et al., 1931). The ratio of carbon dioxide to hydrogen was approximately 2.3 to 1. These ratios were similar for the four isolates tested. Small amounts of ethyl alcohol and pyruvic acid also were detected. Pyruvic acid was identified as the 2,4-dinitrophenylhydrazine derivative (Campbell, 1936; Osburn et al., 1937). Succinic acid (Moyle, 1924) was not detected in any of the D-tartrate fermentations. Neither acetone, isopropyl alcohol, butanol, acetoin, nor 2,3 butanediol could be recovered or demonstrated by qualitative means.

The failure to find succinic acid as one of the end products of the D-tartrate fermentation by these bacteria gives additional support to the belief that previous investigators who reported both succinic and butyric acids as end products of the anaerobic decomposition of tartrate were using mixed cultures. It has been known since 1899 (Grimbert) that the coliform bacteria ferment D-tartrate with the production of succinic acid. (Also see Barker, 1936; Sakaguchi and Tada, 1940.)

Two facts of significance in establishing the mechanism of the D-tartrate fermentation by these bacteria were obtained from these experiments: (1) Small amounts of pyruvic acid were recovered and identified among the end products. (2) The 2.3 to 1 ratio of carbon dioxide to hydrogen gas was found to be almost constant. Proposed steps in the mechanism for the anaerobic decomposition of tartrate by these bacteria, supported by additional quantitative data, will be discussed in a later paper.

Utilization of Tartrate by Other Clostridia

To determine whether the ability to ferment tartrate is a common characteristic of strains of different species of the genus Clostridium, cultures were obtained from sources other than those of the tartrate-fermenting isolates. These included 54 pure cultures representing 25 species of both saccharolytic and proteolytic types. After checking for viability and purity of suspected cultures, they were grown in liver infusion broth and then tested. The test medium contained 0.5 per cent ammonium D-tartrate, 0.3 per cent yeast extract (Difco), 0.5 per cent tryptone (Difco), 0.1 per cent dibasic potassium phosphate, and 0.05 per cent sodium thioglycolate in distilled water. The medium was

adjusted to pH 6.8 with sodium hydroxide, tubed in 200-by-20-mm test tubes filled to two-thirds capacity, sterilized in the autoclave, and inoculated. The inoculum consisted of 10 ml of a 4-day-old liver infusion broth culture of the test organism. The inoculated tubes were incubated at 30 C for 2 weeks and then analyzed for the quantity of unfermented tartrate as compared with uninoculated controls.

With the exception of four strains classified as *Clostridium multifermentans* by Gililland and Vaughn (1943), which actively fermented the tartrate, none of the cultures even caused partial decomposition of the tartrate. On the basis of this experiment it may be concluded that the ability to utilize tartrate readily is not a property common to the majority of strains of the genus *Clostridium*, even of the saccharolytic group.

The Adaptive Character of the Tartrate Fermentation

The realization that the majority of strains of the genus *Clostridium* do not possess the ability to ferment D-tartrate prompted experiments on the adaptive character of that fermentation.

Demonstration of an adaptive enzyme system. Adaptive enzymes in contrast to constitutive enzymes are those enzymes whose production is markedly increased by the presence of a specific substrate. To determine the type of enzyme system involved in the tartrate fermentation an experiment was conducted with an isolate identified as *Clostridium beijerinckii*. The organism was grown in glucose and ammonium D-tartrate media, respectively. The basal medium contained 1.0 per cent tryptone (Difco), 0.5 per cent yeast extract (Difco), and 0.2 per cent dibasic potassium phosphate in distilled water. The glucose medium contained 2.0 per cent glucose. The tartrate medium contained 1.0 per cent ammonium D-tartrate. The two media were autoclaved in glass-stoppered pyrex bottles, then inoculated and filled to capacity with additional sterile solution. After incubation for 20 hours at 30 C the bacteria in approximately 200 ml of the medium were removed by centrifugation, washed once with M/30 Sorensen phosphate buffer (pH 6.8) containing 0.02 per cent sodium sulfide to ensure the reduced conditions necessary for maintaining the activity of the vegetative cells, resuspended in the buffer, and then used.

The experiment was conducted in Thunberg tubes. To one set of tubes was added the same volume of 1.5 per cent sodium potassium D-tartrate solution. Each tube containing substrate or control solutions then received 2 ml of cell suspension. The bacterial suspension was added carefully and in the presence of carbon dioxide, which was blown into the tubes to prevent undue access of air. The tubes were then evacuated. Control tubes for zero time data were heated to kill the vegetative cells, and the remainder incubated at 37 C without shaking. On the termination of incubation (90 minutes) the contents of the Thunberg tubes were heated to destroy the vegetative cells. The cells then were removed from the solutions by centrifugation. The supernatant liquids were decanted and analyzed for glucose and D-tartrate. The results are shown in table 2.

The results show the enzymes involved in the fermentation of D-tartrate to

be adaptive in nature. Only those cells which have grown in the presence of
D-tartrate could ferment it. Since tartrate alone was added to the washed cell
suspensions, the experiment also demonstrates that utilization of D-tartrate by
these organisms involves dissimilation of this substrate alone. The presence of
another organic compound in the medium is not necessary to initiate the break-
down. It also is of interest that the D-tartrate was utilized at a much faster
rate than the glucose.

Adaptation of species of Clostridium to the utilization of tartrate. In view of
the adaptive nature of the enzymes involved in the D-tartrate fermentation an
effort was made to adapt several related species to utilize tartrate. The species
selected were three strains each of *Clostridium butyricum* and *Clostridium bei-
jerinckii* and one strain each of *Clostridium pasteurianum, Clostridium multi-
fermentans, Clostridium acetobutylicum,* and *Clostridium felsineum.* Two methods
were used in the attempt to train the cultures to utilize tartrate. In the first
effort the bacteria were inoculated into a semisolid tryptone medium containing

TABLE 2

Utilization of glucose and tartrate by washed cells of Clostridium beijerinckii (strain O)

SUBSTRATE FOR PRO-DUCTION OF CELLS	SUBSTRATE FOR WASHED CELLS	SUBSTRATE		SUBSTRATE UTILIZED	SUBSTRATE UTILIZED PER ML
		Initial	After 90 min		
		mg/ml	*mg/ml*	*mg*	*micromols*
Glucose	Glucose	4.97	3.57	1.40	7.78
	D-Tartrate	2.96*	3.27	0	0
D-Tartrate	Glucose	4.97	4.05	0.92	5.11
	D-Tartrate	3.02	0.19	2.83	19.1

* As tartrate ion.

0.5 per cent calcium D-tartrate and 0.5 per cent glucose. By repeated transfer
the cultures were kept in active fermentation in this medium for 1 month.
Periodically transfers were taken from the glucose-tartrate medium and placed
in tryptone medium with 0.5 per cent calcium D-tartrate as the only substrate.
The cultures failed to ferment the tartrate. The amount of glucose in the
glucose-tartrate medium was reduced to 0.25 per cent and the same procedure
repeated. Again, none of the cultures could be trained to utilize the tartrate
although the tartrate-fermenting controls always actively fermented the tartrate
in the medium. The second method involved serial transfers of the cultures in
0.5 per cent ammonium D-tartrate, tryptone, yeast extract medium containing
decreasing amounts of glucose (0.3, 0.2, 0.1, 0.05, and 0.00 per cent). This
method also failed.

None of the species tested could be trained to utilize D-tartrate in whole or
in part although the cultures were kept in constant contact with calcium or
ammonium D-tartrate for 6 to 8 months. The addition of acetate to the tar-
trate media did not affect these results (see below).

Utilization of Other Organic Acids

At best the task of determining species allocation of the D-tartrate-fermenting anaerobes was not easy. It was hoped therefore that useful taxonomic data might be obtained by the determination of the ability of these isolates to decompose other organic acids. Fifteen D-tartrate-fermenting cultures were tested for their ability to utilize lactic, succinic, fumaric, L-malic, L-tartaric, malonic, and citric acids in three different semisolid media. The various acids were added to the three basal media containing varying amounts of complex nitrogenous materials, after which the media were adjusted to pH 7.1 with sodium hydroxide and sterilized in the autoclave. Only L-malate was utilized in all three media by 8 of the 15 strains tested. Since D-tartrate is related to D-malate in structural configuration it was thought that D-malate might be utilized by those strains that could not utilize the L-isomer. However, upon inoculation of six of the isolates that did not ferment L-malate into a tryptone, yeast extract medium containing DL-malate, no fermentation was detected after 1 week of incubation.

In a medium rich in complex nitrogenous material qualitative evidence was obtained for the slow fermentation of malonate and other dicarboxylic acids. These studies are being continued.

Clostridium lacto-acetophilum, a species recently described by Bhat and Barker (1947), requires the presence of acetate before it can ferment lactate. Although this anaerobe does not ferment D-tartrate, its other characteristics are similar to those of the 12 tartrate-fermenting isolates identified as *Clostridium butyricum*. It was desirable therefore to determine the effect of acetate on the ability of the tartrate-fermenting strains to utilize lactate. Eight strains were tested in two media. One contained lactate alone; the other contained lactate and acetate. The basal medium contained 0.5 per cent yeast extract (Difco), 0.1 gram dibasic potassium phosphate, 0.3 per cent agar, and traces of ferrous, manganous, and magnesium salts. Lactate and acetate were added in concentrations of 0.5 per cent. The media were sterilized and inoculated as previously described.

The results of the experiment were clear-cut and uniform. In the medium containing lactate alone the bacteria showed a slight activity, whereas in the medium with lactate plus acetate a vigorous fermentation was observed. This type of fermentation may be one possible explanation of the positive results obtained with malonate, as mentioned above. Acetate or some other compound that can perform the same function as acetate in the fermentation of lactate by these clostridia may be present in the medium.

The addition of acetate, however, did not influence the ability of the tartrate-fermenting cultures to decompose the other dicarboxylic acids. Those strains that failed to ferment succinate, fumarate, and L-malate alone did not ferment these substrates in the presence of acetate.

REMARKS ON SPECIES ALLOCATION

As previously stressed, the D-tartrate-fermenting cultures in general had characters that related them to *Clostridium butyricum*. Some of the strains

differed in their ability to utilize one or more carbohydrates. By following Spray (1948) in *Bergey's Manual of Determinative Bacteriology* it was possible to allocate all but two of the isolates to one of three species: *Clostridium butyricum*, *Clostridium beijerinckii*, or *Clostridium pasteurianum*. Two of the isolates differed significantly from other well-described species and could not be classified.

If the isolates had been similar enough to have been identified as belonging to one species, it would have been possible to name a new species or subspecies based on their unique ability to ferment D-tartrate. The cultures, however, could be allocated to several recognized species. Therefore, creation of a new species or subspecies is not justified, even for the purpose of serving to identify or emphasize tartrate-fermenting butyric acid bacteria of the type described. To do so would give unwarranted prominence to the tartrate-fermenting ability of the bacteria and lead directly to further confusion in the taxonomy and nomenclature of the saccharolytic species of *Clostridium*.

Enrichment cultures containing as yet untried substrates, when inoculated with material from proper sources, undoubtedly will yield many more types of clostridia than now recognized. If the ability to utilize these untried substrates were given undue importance, as for example could be given to D-tartrate fermentation, in order to classify all of the isolates, it is obvious that an infinite number of new species and varieties soon would be created.

When confronted with a similar type of problem with the coliform bacteria, Levine (1918) chose those few differential characters that careful statistical analysis had shown would give good correlative relationships. This method has resulted in a significant reduction in the number of differential tests necessary to identify the individual species. Perhaps a similar approach to the taxonomy of the saccharolytic bacteria of the genus *Clostridium* would reduce the present number of species and simplify the identification and classification of new isolates.

SUMMARY

The characteristics of 23 D-tartrate-fermenting butyric anaerobes isolated from spoiled tartrates, tartrate recovery equipment, and vineyard soils are given. All but two isolates were tentatively identified as representing previously described species closely related to or identical with *Clostridium butyricum*.

The major end products of D-tartrate fermentation by representative cultures included acetic acid, butyric acid, carbon dioxide, and hydrogen. The molar ratio of acetic acid to butyric acid formed was approximately 10:1. Small amounts of ethyl alcohol and pyruvic acid were formed.

The enzymes involved in the decomposition of D-tartrate were shown to be adaptive in character. Attempts to adapt other cultures of the common saccharolytic species of *Clostridium* to the utilization of D-tartrate were unsuccessful. The ability to ferment D-tartrate is not general among the representatives of the genus *Clostridium.*

With the exception of L-malic acid, four carbon dicarboxylic acids other than D-tartaric were not readily attacked by the bacteria under the conditions of the experiments.

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THE ISOLATION OF L TYPE CULTURES FROM BACTEROIDES WITH THE AID OF PENICILLIN AND THEIR REVERSION INTO THE USUAL BACILLI[1]

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It has been reported previously (Dienes and Smith, 1944) that in cultures of certain strains of *Bacteroides* colonies develop which in appearance, morphology, and properties of growth are similar to L_1 colonies in *Streptobacillus moniliformis*. These colonies develop from the bacilli in a similar manner in both species. The bacilli swell into large fusiform or round bodies, which grow either into L type colonies or return to the usual bacilli. Klieneberger (1948) has examined one of the *Bacteroides* strains studied by Smith and the author, and she observed both the similarity between the L type colonies of *Bacteroides* and those of *Streptobacillus moniliformis* and the derivation of these colonies from swollen bacterial forms.

The study of L type colonies is more difficult in *Bacteroides* than in *Streptobacillus moniliformis* because they develop only in the cultures of a few exceptional strains and only for a short period following isolation. Of 23 strains of *Bacteroides* studied in this laboratory, 9 were pleomorphic, and L type colonies were observed in only 4. They could be isolated in pure culture from 2 strains only, and only by keeping the cultures frozen in CO_2 ice was it possible to preserve their tendency to produce such colonies. The cultures of nonpleomorphic strains and of those which lost this property during cultivation consist of regularly shaped bacillary forms without any morphological peculiarities.

Shortly after penicillin became available Pierce (1942) observed that L_1 is highly resistant to it and can be readily isolated in pure culture by inoculating *Streptobacillus moniliformis* on media containing the antibiotic. Attempts made at that time by the author to isolate L type colonies from other bacteria with the help of penicillin were not successful. During the last two years, by studying the effects of high concentrations of penicillin and by using various media, the development of L type colonies has been observed in many species, and from some species it has been possible to isolate these forms in pure culture. This paper contains observations made with *Bacteroides*; those made with *Hemophilus influenzae* have already been briefly reported (Dienes, 1947b). According to the observations to be described, the addition of penicillin to the medium offers an easy method for the isolation of L type colonies from certain strains of *Bacteroides* in the same way as from *Streptobacillus moniliformis*.

However, these observations imply more than the development of a new technical procedure; they indicate an unexpected effect of penicillin on bacteria. Penicillin apparently helps the isolation of L type colonies not only by suppression of bacterial growth but also by actual induction of the development of L forms in cultures in which they would not otherwise be seen.

Six strains of *Bacteroides* were used for the study of the effects of penicillin. One strain (132) had already been studied extensively. Two cultures of this strain were available: one which had been preserved in CO_2 ice and had remained pleomorphic, and another which had lost its pleomorphism as a result of continued transfers in broth. The second strain (Ph) was cultivated from a chronic infection of the knee joint. It was not pleomorphic and produced no L type colonies. The third strain (224) was isolated from a fistula of the abdominal wall and was also nonpleomorphic. Three strains (906, 133, and 701) were received from the Boston City Hospital through the courtesy of Miss Lamb. They were maintained by passages in meat tubes, and none of them produced L type colonies. All strains were gram-negative, nonmotile, nonsporeforming, anaerobic rods, and they were all isolated from suppurative processes in humans. To attempt further classification has not been regarded as necessary because at present it is impossible to decide whether the differences between the strains really indicate difference of species. Pleomorphism is a transitory characteristic in most strains and certainly does not indicate difference of species.

Thioglycolate broth cultures of the strains were transferred to agar plates containing concentrations of penicillin increasing from 1 to 5,000 units per ml. The medium was similar to that employed for isolation of pleuropneumonialike organisms. It is prepared by boiling nutrient agar with 2 per cent horse blood. After sedimentation of the precipitate, the supernatant is mixed with 20 to 30 per cent ascitic fluid. Anaerobiasis was produced as before by growing *Serratia marcescens* on a piece of agar on the lid of the sealed plate. Results obtained with the different strains varied to a great extent.

The first two strains (132 and Ph) produced L type colonies abundantly on penicillin plates. Bacterial growth was prevented by the smallest dose of penicillin tested (1 unit per ml). The bacteria transferred to the plates swelled up within a few hours into large round bodies, and L type colonies developed in the course of 2 to 3 days even with the highest concentrations of penicillin (5,000 units per ml). With strain 132, it made no difference whether the plates were inoculated with pleomorphic or nonpleomorphic cultures. Between September, 1947, and February, 1948, six experiments were conducted with these strains. The development of L type colonies varied considerably, but they grew in one or more plates in each experiment, more regularly in those with the highest concentrations of penicillin. In certain experiments some plates remained sterile while there was growth in others. For example, L type colonies developed abundantly in an experiment with 100 and 1,600 units and they were absent with 200 and 400 units. The variation is probably due in part to technical difficulties of anaerobic cultivation, and its cause was not further studied. The appearance of the plate with dense growth of tiny colonies and the structure of

the colonies as they appear unstained on the agar with moderate magnification is shown in photographs 5 and 8 (figure 1).

The colonies were transferred by cutting out a square of agar containing them and streaking it over the new medium. They grew equally well on media with or without penicillin. The agar squares are left on the fresh medium because growth often develops only beneath the square. On one occasion when transplants were made from a plate containing 1 unit of penicillin per ml, a few bacterial colonies developed in addition to L type ones. However, the latter type of colonies grew in transplants made from plates with higher concentrations of penicillin, and bacterial colonies did not develop either in the primary or in the subsequent transfers on agar. The number of colonies on ascitic agar is always low, although the colonies develop to a considerable size (1 to 1.5 mm) after 7 to 10 days' incubation. The most abundant growth can be obtained by restreaking the agar squares over the medium after 2 to 3 days' incubation and reincubating the plates. After 24 hours' incubation the colonies are very small and entirely embedded in the agar.

A few L type colonies developed from two of the strains received from the Boston City Hospital. Strain 906 produced a few bacterial colonies consisting of long filaments and a few L type colonies on plates containing 1 unit of penicillin. No growth was present with higher concentrations. Strain 133 produced a slight bacterial growth with 1 unit of penicillin per ml. Growth was absent with higher concentrations, but a few L type colonies developed with 50 units per ml. No L type colonies were obtained from these strains in two subsequent experiments. The third strain (701) received from the City Hospital and strain 224 isolated in our laboratory produced no L type colonies in two consecutive tests. In all experiments an area of the agar was inoculated with cultures of strain 132, which produced L type colonies abundantly in most of the plates.

The *Bacteroides* strains present a distinct individuality manifested in different degrees of pleomorphism, or in its absence, and in the tendency to produce L type colonies. The individuality of the strains is as marked with penicillin as without it. In some strains of *Bacteroides* penicillin induced an abundant growth of L type colonies; in others, only a slight growth or none at all. In the present experiments only one medium, ascitic agar, was used. Observations with other bacteria indicate that the composition of the medium and the origin of the serum added to it exert a marked influence on the results. The medium which was most successful with *Proteus* and *Eberthella typhosa* was a soft agar to which 10 per cent horse serum had been added. This medium also yielded a more abundant growth of L type colonies in *Bacteroides* than ascitic agar. It is probable that a large percentage of *Bacteroides* strains could be induced to produce L type colonies by experimenting with various media.

The L type colonies isolated with or without penicillin are similar in every respect. The appearance and growth of these colonies and the morphology and physical properties of the organisms are so characteristic and differ in so many respects from the parent culture that their identification causes no difficulty. The close similarity of these colonies to the L_1 was acknowledged by Kliene-
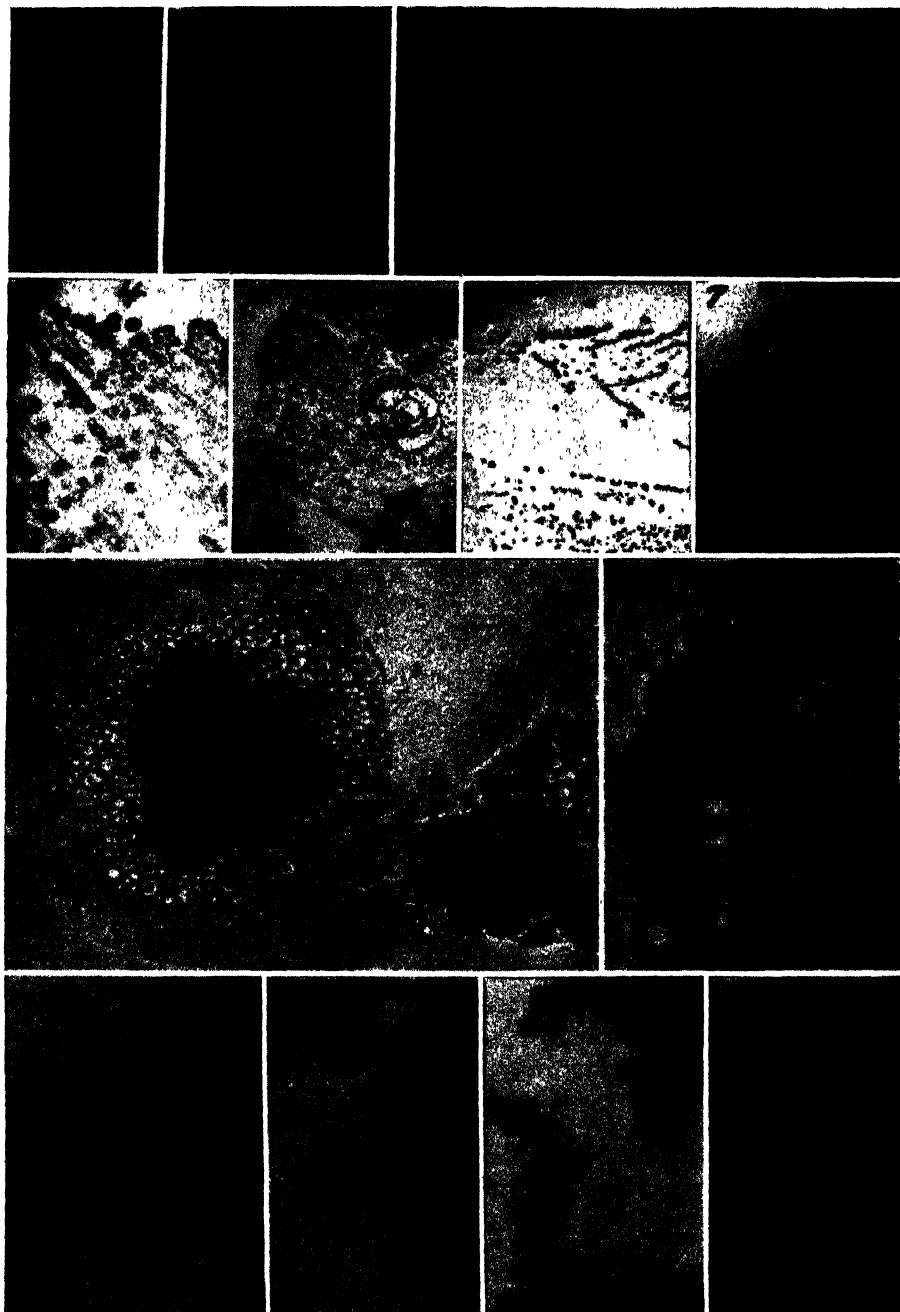
Figure 1

1. A nonpleomorphic broth culture of *Bacteroides* strain 132. × 2,000.
2. A culture similar to that in photograph 1 after 5 hours' exposure to 400 units penicillin er ml. × 2,000.

berger, and it is apparent in the photographs in figure 1. As in the case of L_1 (Dienes, 1947c), it is difficult to see and especially to photograph the shape of the small growing forms in the center of the colonies since they do not grow in one plane and cannot be transferred to a cover slip. In a successfully stained agar preparation very small bacilli, often with polar staining, and all transitional forms to the very large round bodies are clearly visible. Photographs 7, 10, 11, and 12 were made from the extending edge of the dense center of the colonies. Photograph 13 was made from an impression preparation of colonies from which the surface layer had been removed. Although the shape of the individual organisms cannot be seen so clearly in the photographs as would be desirable, both small bacillary and rounded forms are apparent. The shape and arrangement of the organisms are similar to those visible in photographs of L_1 and of pleuropneumonialike organisms made from similar preparations. The author cannot agree with Klieneberger's opinion that these organisms show no "cell boundaries" and grow as "thin shapeless slime." The form of the organisms is as distinct as the form of any other microorganisms, and their development follows a definite pattern.

The growth properties of the L type colonies isolated from *Bacteroides* and the L_1 are similar both on solid and liquid media. It is difficult to induce growth, and on a heavily inoculated plate only a few organisms will produce colonies. These develop slowly, but if they are not crowded they continue to grow for 5 to 7 days. Autolysis is often apparent, although the colonies continue to extend to the periphery. In broth, growth develops slowly and continues for several weeks as isolated colonies adhering to the sides of the tube, or in thioglycolate broth adhering to the agar particles.

Some insight into the mechanism by which penicillin exerts its influence on

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3. Transplant on ascitic agar from culture illustrated in photograph 2 after 17 hours' incubation. $\times$ 2,000.

4. Bacillary colonies of strain 132 on ascitic agar. $\times$ 2.

5. Tiny L type colonies on 10 per cent horse serum agar containing 1,600 units penicillin per ml inoculated with a nonpleomorphic culture of *Bacteroides* strain 132. Three-day-old culture. $\times$ 2.

6. L type colonies of strain 132 after the 15th passage on ascitic agar. Three-day-old culture. $\times$ 2.

8. L type colonies of strain 132. They have a dense center embedded in the medium with granular appearance and irregular contour. The periphery of the colony extends on the surface of the medium consisting of large round forms. These are apparent with this magnification only when they are transformed into large vacuoles. $\times$ 200.

9. The periphery of a colony similar to that in photograph 8. Many of the round bodies are not stained and the one in the lower left corner has a round vacuole. $\times$ 2,000.

7, 10, 11, and 12. These photographs illustrate the edge of the center of L type colonies spreading in the agar. This consists of small bacillary forms and small and somewhat larger round forms arranged in a way similar to bacilli growing into the agar. The short filaments with a knob or round swelling at the end can be seen often in cultures of pleuropneumonialike organisms. The organisms in the photographs are in their natural arrangement, although they are flattened by the drying of the agar. Photographs 7, 11, and 12, $\times$ 3,000; 10, $\times$ 2,0000.

13. Impression preparation after agar fixation stained with thionin. The surface of the colonies was eliminated and the impression made from the dense center. All transitions from very small to large round forms are present. $\times$ 3,000.

Photographs 1, 2, 3, 5, and 9 were made from wet-stained agar preparations; 7, 10, 11, and 12 from dry-stained agar preparations; 13 from an impression preparation after agar fixation. Photographs 4, 5, 6, and 8 were photographed from unstained agar plates.

the bacilli was obtained by study of the broth cultures of strain 132. In broth cultures of this strain under normal conditions of cultivation, pleomorphism is the result of the changes induced by the growth of the bacillus in the medium. The bacilli are not pleomorphic on solid media, and transferred into broth they start to multiply in the form of regularly shaped bacilli and short filaments. After 6 to 9 hours' incubation almost all bacilli develop central swellings at the same time, and during the following hours swell up to large bodies. Multiplication in bacillary form stops during this process. If the large bodies are transferred into fresh broth or agar at any time during the course of their development, they break up into small bacillary forms. They produce no pleuropneumonialike colonies. After the transformation of the bacilli into large bodies is complete in the broth cultures, both bacillary and L type colonies develop in transplants and the proportion of pleuropneumonialike colonies increases with the age of the cultures. The peculiar processes observed in the cultures are the response of the bacilli to the accumulation of metabolic products in the medium.

The culture of strain 132 that was used for the following experiments was propagated by passage in thioglycolate broth and lost its pleomorphism and ability to produce L type colonies. Throughout its whole development it consisted of small bacillary forms. To obtain a fresh growing culture, thioglycolate broth containing ascitic fluid was heavily inoculated with this strain and incubated for 3 hours. Increase of turbidity and gas production indicated vigorous growth. At this time enough penicillin was added to the cultures to bring the concentrations up to 100 and 400 units, respectively. Gas formation and increase of turbidity proceeded during the following hours, but were caused by an increase in the size of the bacilli and not by multiplication. Five hours after the addition of penicillin no usual bacilli were visible in broth; they had been replaced by large round bodies. At that time transfers were made on two ascitic agar plates. After incubation overnight one plate was opened, and the large bodies were found in all stages of transformation into bacilli. It can be seen in photograph 3 that the large bodies increased in size; they began to segment and bacillary filaments began to grow from them in multiple directions. Under the microscope this plate presented a picture similar to that observed in plates inoculated from young broth cultures of naturally pleomorphic strains. On the plate opened after 48 hours bacillary colonies were present in large numbers without admixture of L type colonies. Transferred to broth, these bacilli produced no pleomorphic forms.

The same broth cultures were transplanted after 2 days' exposure to penicillin. The large bodies were well preserved and stained deeply with methylene blue, but, instead of developing into bacilli, they produced L type colonies exclusively. These colonies grew well in transplants on ascitic agar plates both with and without penicillin. The appearance of the cultures was similar to that reproduced in photographs 5 and 8. The derivation of the L type colonies from the large bodies has previously been demonstrated by observation of this process in slide cultures. This method cannot be applied so well to large bodies produced by penicillin, because at the stage when they grow into L type colonies

only a few of them remain viable. However, in stained agar preparations it could be seen that in this case also the L type colonies came from the large bodies. The broth cultures have no other growing organisms besides the bacilli and the L type colonies evidently originate from them.

The influence of penicillin on nonpleomorphic broth cultures of strain 132 imitates closely the processes observed in pleomorphic broth cultures without penicillin. In both cases the swelling of bacteria into large forms and the growth of L type colonies are a response of the bacteria to the presence of chemical substances in the medium interfering with normal growth. This observation is of great interest because it indicates that the mechanism by which penicillin induces the production of L type colonies is probably closely similar to that by which these colonies are produced in pleomorphic cultures without penicillin.

The L type cultures isolated some years ago from strain 132 and from another pleomorphic strain O.H. could not be made to grow in liquid media. The cultures isolated with the help of penicillin both in strains 132 and Ph grew in thioglycolate broth containing ascitic fluid. This medium had not been used previously. To make a culture in broth, the colonies together with the superficial layer of medium were scratched off the surface of agar cultures and transferred into thioglycolate broth. The cultures were then sealed with liquid petrolatum. The colonies adhering to the agar continued to enlarge during the following days, and the metabolic activity of the culture was indicated by a slight development of gas. It was helpful at this point to break up the colonies by drawing them into a capillary pipette several times. In some cases, following this procedure, several hundred small colonies developed adhering to the agar suspended in the medium. The colonies were small but easily seen. Examined under the microscope they consisted of small to very large round bodies (up to 20 to 30 μ) most of which were empty and transparent; some were filled with granules. Similar empty blebs develop in cultures of the pleuropneumonia group of organisms and they are probably not living structures. The center of young colonies consisted of small forms. Growth was obtained in successive transfers in thioglycolate broth, but it was always slow and the gas production remained slight compared with that in bacillary cultures.

The most important observation made with the broth cultures was that sooner or later the usual bacillary forms reappeared in them. This observation is of considerable interest for the question of the nature of the L type growth, and one experiment will be described in detail.

L type colonies from penicillin plates inoculated with bacillary cultures were transferred on December 22 to ascitic agar plates without penicillin from both strains 132 and Ph. The cultures were transferred by cutting out agar squares containing the colonies and streaking them on fresh plates. Transfers were made at intervals of 3 to 5 days. The usual bacillary forms never appeared on the plates. Both strains were transferred from the agar plates into two thioglycolate broth tubes containing 10 to 20 per cent ascitic fluid from the 2nd, 3rd, 5th, 8th, 13th, 14th, and 15th passages on agar. The last transfer into broth was made on February 14, 54 days after the original isolation of the L type

growth. The broth tubes were stirred after 4 to 5 days by a capillary pipette, and at the same time transfers were made into fresh thioglycolate ascitic fluid tubes. Development of bacilli in the broth cultures was indicated by abundant gas formation and turbidity. The bacilli were examined for their morphology and growth under aerobic conditions. In all cases they presented the pleomorphism characteristic of certain freshly isolated strains of *Bacteroides*. This pleomorphism and the absence of growth under aerobic conditions is sufficient to identify them. A few cultures were more thoroughly examined and were found to be similar in every respect to the original strains. On agar they produced L type colonies in addition to bacillary ones. Contamination of the culture occurred only in 5 tubes out of the 43 used, both in original and secondary transplants. The contaminants did not produce gas in thioglycolate broth and grew in aerobic plates.

In table 1 are indicated the dates when bacilli were noticed in the broth cultures. They appeared in the tubes inoculated from the agar cultures after 4 to 33 days of incubation. Twenty-three transplants were made from the original tubes before bacillary growth was apparent; two were contaminated, but sooner or later bacilli developed in all the others. In some cases this happened only after long incubation of the subculture, in one instance after 64 days.

It is of interest that the bacilli which appeared in broth were pleomorphic in all cases and that after growth overnight the culture consisted of large round bodies and short filaments with large swellings. The cultures of strain 132, which had lost its pleomorphism, and strain Ph, which had never shown pleomorphism, both acquired a tendency to swell into large bodies similar to that observed in strain 132 immediately after isolation. One of the 132 cultures regained from L type colonies was propagated in broth cultures. The pleomorphism persisted in 5 consecutive transfers and disappeared in the sixth. This behavior is similar to that of the original strain. It has already been mentioned that the bacilli regained from the large bodies without passing through the L forms were not pleomorphic. Exposure to penicillin and to certain toxic salts induces the swelling of bacteria into large bodies, but these forms transferred on normal media reproduce bacteria of the usual morphology if they multiply at all. The passage of bacteria through the L forms is the only instance known to the author in which a tendency to pleomorphic growth, which persisted in successive generations, has been produced.

The reappearance of bacillary forms in the broth cultures is certainly not a result of contamination. The bacilli which reappeared were similar in every case, and the characteristic pleomorphism, anaerobic growth, and the production of L type colonies after transfer to agar proved their identity with the parent strain. The transfers and the examination of the cultures were always made by the author, and the media were tested for sterility. The thioglycolate broth was boiled immediately before inoculation. The ascitic fluid was also used for the cultivation of pleuropneumonialike organisms and of such routine specimens as joint and pleural fluids. *Bacteroides* never appeared in these cultures. At the same time that the thioglycolate tubes were inoculated from the third

transfer of L type colonies, two tubes were inoculated as a control with cultures of L_1 isolated several months before from a strain of *Streptobacillus moniliformis*. These tubes were stirred and transferred into two other tubes 3 days later. During the following days *Streptobacillus moniliformis* grew in all four tubes.

TABLE 1

Development of the usual bacilli in thioglycolate broth inoculated with L type cultures of Bacteroides strains 132 and Ph

NUMBER OF PASSAGES ON ASCITIC AGAR PLATES WITHOUT PENICILLIN BEFORE INOCULATION INTO BROTH (L TYPE CULTURES)	DATE OF TRANSFER INTO BROTH	DATE OF APPEARANCE OF BACILLI IN THE BROTH CULTURES	
3	Jan. 5	Strain 132	Jan. 10
		132	Jan. 10
		Ph	Jan. 10
		Ph	Jan. 12
5	Jan. 13	132	Jan. 24
		132	Jan. 31
		Ph	Jan. 19
		Ph	Jan. 20
8	Jan. 24	132	contam.
		132	Feb. 5
		Ph	Jan. 28
		Ph	Feb. 1
13	Feb. 14	132	Mar. 11
		132	Mar. 18
		Ph	contam.
		Ph	Feb. 26
15th (strain 132)	Feb. 26	132	Mar. 4
		132	Mar. 15
14th (strain Ph)		Ph	contam.
		Ph	Mar. 20

The broth cultures were stirred and transferred into other broth tubes after 3 to 6 days' incubation. Bacilli appeared in only one tube before stirring (Jan. 28, 8th passage of strain Ph). Bacilli appeared in 21 of 23 thioglycolate tubes inoculated from the original broth cultures; 2 tubes were contaminated.

The four thioglycolate tubes inoculated simultaneously with L type colonies of *Bacteroides* later produced *Bacteroides*. The L type colonies in both cases reproduced the bacteria from which they originated.

DISCUSSION

L type colonies have been obtained without difficulty with the help of penicillin from certain strains of *Bacteroides*. The author hopes that the easy availability of these peculiar forms will attract others to their study. A high re-

sistance to penicillin is one more similarity between these colonies obtained from *Bacteroides* and the L_1 isolated from *Streptobacillus moniliformis*. The growth of L type colonies was not affected in either species by the highest concentrations of penicillin tested, 5,000 and 10,000 units, respectively. On the other hand, these colonies grew as well without penicillin. It is interesting that in both species this high resistance to penicillin is connected with colonies showing a close similarity in the type of growth on agar and in broth, in morphology and physical properties. However, metabolic activities of the L type cultures are different in the two species and similar to that of the parent organism, although in both cases they are greatly reduced in intensity.

The fact that penicillin favors the development of L type colonies in *Streptobacillus moniliformis* and *Bacteroides* is of considerable interest in connection with observations made with other bacteria. The isolation of colonies corresponding to the L_1 from *H. influenzae*, *E. typhosa*, and *Proteus* with the help of penicillin will be the subject of future papers. In these cases L type colonies could not be isolated without penicillin, although they are apparent in some cultures in a rudimentary form. If the L type colonies developing with or without penicillin are similar in two species, there is no reason to believe that in other species colonies with similar properties are of a different nature simply because at present they can be obtained only by the use of penicillin. The inducement of the growth of L type colonies is an unexpected effect of penicillin. This effect certainly represents more than mere inhibition of bacterial growth, but, since most of the information concerning it was obtained from the study of other species, its probable nature will be discussed in future publications.

The reappearance of bacilli in broth cultures of L forms of *Bacteroides* is important in ascertaining the nature of these forms, and it is an additional similarity between these forms and the L_1. The observations made with *Bacteroides* are similar to those reported by various authors on L_1 strains. Heilman (1941) studied several L_1 strains which returned to bacillary forms in broth during a certain period after isolation. Often several transfers in broth were needed for this purpose. The L_1 strain studied by Brown and Nunemaker (1942) returned in broth to the bacillary form after 120 transfers on solid media. Klieneberger (1940) observed the reappearance of bacilli in broth cultures, but she stressed the fact that after prolonged cultivation on solid media many strains became stabilized and lost the ability to return to the bacillary form. It is not apparent in the present experiments whether a similar stabilization in the L form occurs in *Bacteroides*.

Klieneberger tries to explain the reappearance of the bacilli in broth by the hypothesis that the bacilli are carried in the L_1 colonies as a contamination. A critical examination shows that this supposition does not agree with the observations. Both in *Streptobacillus moniliformis* and *Bacteroides* the L forms were transferred over long periods in pure culture on solid media, a fact which excludes the possibility that bacteria accidentally included in the cultures would persist without multiplication. The bacilli certainly do not multiply in the L type colonies in their usual form, because they do not form colonies on agar and

require long incubation in broth in order to be apparent. In both species the bacilli grow much faster than the L type cultures. The author has discussed the connection between the bacillus and the L_1 elsewhere (1947a). It is sufficient to say here that the L forms of *Bacteroides* are not only similar morphologically to the L_1 and that they are derived in a similar way from the bacilli, but that they return to the bacillary form in a similar manner. The L forms behave in both species as variant forms and not as organisms genetically different from the bacilli.

SUMMARY

The growth of six *Bacteroides* strains was studied on ascitic agar plates containing from 1 to 5,000 units of penicillin per ml of the medium. Growth in the usual bacillary forms was inhibited and L type colonies developed in abundance in two strains with all concentrations of penicillin. A few such colonies developed in plates inoculated with two other strains and none on those inoculated with the remaining two strains. The individual peculiarities of the strains in the development of L type colonies are as marked with penicillin as without it. The influence of the composition of the media in the development of L type colonies was not studied.

The processes induced by penicillin resulting in the growth of L type colonies are similar in many respects to those observed in pleomorphic strains without the use of penicillin. The bacilli, in a culture of the previously studied strain 132 after loss of pleomorphism, were induced by penicillin to swell to large forms. In the early stages of transformation these large forms transferred to penicillin-free media returned to the usual bacillary form; in a later stage they produced only L type colonies.

The L type colonies of two strains were induced to grow in thioglycolate broth. After prolonged incubation (4 to 64 days), the usual bacilli reappeared in all broth cultures. On agar plates development of bacilli was never observed in L type cultures. Before the L type cultures were inoculated into broth, they were transplanted on agar for a sufficient number of times to exclude the presence of bacilli accidentally included in them. The L type of growth in *Streptobacillus moniliformis* and that in *Bacteroides* are similar not only in their derivation from bacilli, in morphology, and in high resistance to penicillin, but also in the fact that, under appropriate conditions, they reproduce the parent bacilli in a similar way.

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MUTATION IN STREPTOMYCES FLAVEOLUS INDUCED BY X-RAYS AND ULTRAVIOLET LIGHT[1,2]

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The actinomycetes have been the subject in recent years of fundamental cytological and physiological studies. But there has been little quantitative genetic study of this difficult group of microorganisms. The voluminous literature on spontaneous variation in actinomycetes will not be reviewed here. There have apparently been no studies of induced mutation in actinomycetes, such as exist for the fungus *Neurospora crassa* (Sansome *et al.*, 1945; Hollaender *et al.*, 1945), or the bacterium *Escherichia coli* (Demerec and Latarjet, 1946).

The present study had three general aims: (1) Determination of the comparative usefulness of ultraviolet light and X-rays as tools for inducing mutation in actinomycetes. (2) Determination of the irradiation doses giving maximum mutation frequency. This knowledge was desired for a study of the genetics of antibiotic formation in actinomycetes (Kelner, unpublished work). (3) Study of the types of morphological mutants most easily induced in actinomycetes. Mutation causing change in gross morphology was selected as the type to be studied, because such mutations were likely to be more abundant than were other types.

MATERIAL AND METHODS

One species, *Streptomyces flaveolus* ATCC 3319, was chosen as a representative actinomycete species. Its free sporulation made it easy to prepare conidial suspensions. According to Bergey *et al.* (1939), it is supposed to produce abundant yellow soluble pigment in organic media. Mutations affecting pigmentation were expected to be especially striking and easily scored. The culture as received from the American Type Culture Collection, however, produced no, or only faint yellow, pigment—a not unexpected departure from description for actinomycetes. The lack of pigmentation proved not to be serious, for the most common mutant induced was an easily scored yellow form.

A single colony isolate of *S. flaveolus* was used to inoculate 40 asparagine glucose agar sporulation slants.[3] These were incubated 2 weeks at room temperature, then stored at 5 C. Conidia for all experiments came from two such stocks of slants.

The ultraviolet source was a General Electric 15-watt germicidal lamp, 80 per cent of whose radiation was at 2537Å. A constant voltmeter maintained the voltage at 112 volts during irradiation. The lamp was calibrated by determining

the survival curve of coli phage T2 (Latarjet and Wahl, 1945; Luria and Latarjet, 1947). All irradiations were made 60 cm distant from the lamp, at an intensity of 16 ergs $\times$ sec^{-1} $\times$ mm^{-2}.

Suspensions were X-rayed at the Physics Department of the Memorial Hospital, New York.[4] The X-rays were produced at 180 Kv. The rays were unfiltered, and had a H. V. L. of 0.19 mm Cu. The intensity as measured in air was 2,000 roentgens per minute.

Preparatory to irradiation spores of S. flaveolus were washed off the agar slant into sterile saline. The resulting suspension was filtered 6 times through absorbent cotton, and finally brought to a turbidity averaging about 1×10^7 spores per ml. Ninety per cent of the particles in the filtered suspension were single conidia (or possibly chains of two closely linked conidia).

For experiments with ultraviolet irradiation, 15 ml of the suspension were placed in a round-bottomed 100-ml flask of ultraviolet-transparent vitreous silica.[5] During the irradiation the flask was rotated at 330 rpm, to ensure uniform exposure of the spores (a method adapted from Hollaender and Emmons, 1946).

Suspensions for X-ray treatment were prepared similarly, except that 1 part nutrient broth to 9 of the suspension was added before irradiation, in order to obviate secondary lethal effects caused by "activated water" (Lea, 1947). Suspensions were X-rayed in small pyrex tubes. In all X-ray experiments, suspensions were prepared in the afternoon, kept at 0 C overnight, X-rayed the next morning in New York (30 miles from the laboratory), and plated on the nutrient agar 2 or 3 hours after irradiation. In ultraviolet experiments, however, suspensions were prepared immediately before, and seeded immediately after, irradiation.

The irradiated spores were plated on nutrient agar plates by spreading 0.1 ml of appropriate dilutions over the agar surface with a bent glass rod. After incubation for 3 days at 28 C, 100 colonies selected at random were each spread over an area 2 to 3 cm^2 on asparagine glucose agar plates and incubated 1 week at 28 C. Each inoculum grew as a confluent mass. On this medium differences in pigmentation, sporulation, and growth vigor were far more striking than on nutrient agar. Mutants were scored according to their growth on the asparagine agar plates, inoculated as described, and incubated 1 week at 28 C. If irradiated cells were plated directly to asparagine glucose agar, survival ratios were very variable. The method used had a further advantage; the mutants on the asparagine agar were at least two generations removed from the irradiated cell, and hence the observed morphological differences were clearly inheritable.

Controls of nonirradiated conidia were plated exactly as were irradiated conidia.

EXPERIMENTAL DATA

Mutant types. The nonmutated parent or wild type of S. flaveolus 3319, with which all mutants were compared, had moderate, flat, whitish vegetative growth

[4] With the kind assistance and advice of Miss Elizabeth Focht and Dr. Seymour Wollman of that institution.

[5] Manufactured by Vitreosil Company, New York.

and abundant gray spores. The reverse was gray to white, or extremely faint, drab yellow. There was evident no yellow pigmentation in the agar, except when the agar was viewed through its greatest depth (as from the side of the plate), whereupon a very faint yellow pigmentation was sometimes seen.

The *yellow* mutant had a moderate to abundant, often raised, vegetative growth and moderately abundant, chalk-white spores. The reverse was a moderate to intense yellow, with occasional red-orange areas. There was a vivid aura of yellow pigmentation in the agar, easily visible through a thin layer of agar (as when the petri dish was viewed from above). The spores of some strains became faint gray or cinnamon-colored.

The *asporogenous* mutant was characterized by a complete absence of aerial spores, or at most a very faint, questionable sporulation. It should be emphasized that some *asporogenous* mutants may have sporulated if incubated longer or if cultured on media other than asparagine agar (e.g., one strain sporulated well on nutrient agar but not on asparagine agar; the parent strain sporulated well on both media).

The *restricted* mutant showed only trace growth on asparagine agar.

Strains differing in other ways from the parent type were always present—e.g., strains with partially reduced sporulation, or growth vigor; and strains with dark gray or tan reverse. Although many such strains were undoubtedly mutants, they were all scored as the wild type because of the difficulty in differentiating them from the parent.

The relative frequency of mutant types was *yellow, restricted*, and *asporogenous*, in decreasing order. These strains were found in both ultraviolet-irradiated and in X-rayed cultures. The yellow mutants that did appear in the controls were in all cases pale yellow, none having the deep orange-yellow pigmentation of many of the yellow mutants in irradiated cultures.

Nonirradiated control cultures had a low incidence of mutation, 1.0 per cent; 8 yellow, 1 restricted, and no asporogenous mutants were present among the total 938 control strains scored. A total of 2,432 strains from irradiated conidia were scored to obtain the mutation-frequency data given later.

Experiments with ultraviolet irradiation. Figure 1 shows the survival of *S. flaveolus* conidia at various doses of ultraviolet radiation. The survival curve is essentially exponential except in the beginning (to a survival of 20 per cent), where the rate of killing is less than in the later portions of the curve. A similar upward bend in the first part of the survival curve has been noted in *Trychophyton mentagrophytes* (Hollaender and Emmons, 1941), *Aspergillus terreus*, (Hollaender et al., 1945) and *Escherichia coli* (Demerec and Latarjet, 1946) and may be due to the presence of clumps or small chains of cells. *S. flaveolus* was apparently far more resistant to ultraviolet irradiation than was *Escherichia coli* (Demerec and Latarjet, 1946) and had the same order of resistance as many fungus spores (Hollaender, 1942). Valid generalizations cannot yet be made of the relative resistance to ultraviolet light of various groups of microorganisms, because too few species have been quantitatively studied.

The mutation frequency as related to ultraviolet dose in four separate experiments is shown in figure 2.

A control was run for each suspension in order to determine the percentage of spontaneous mutants. The spontaneous mutation frequency varied in different suspensions, ranging from 0 to 2 per cent. The induced mutation frequency of irradiated cells at any particular dose shown in the graph has been corrected for the spontaneous mutation frequency of the suspension used in the individual experiment.
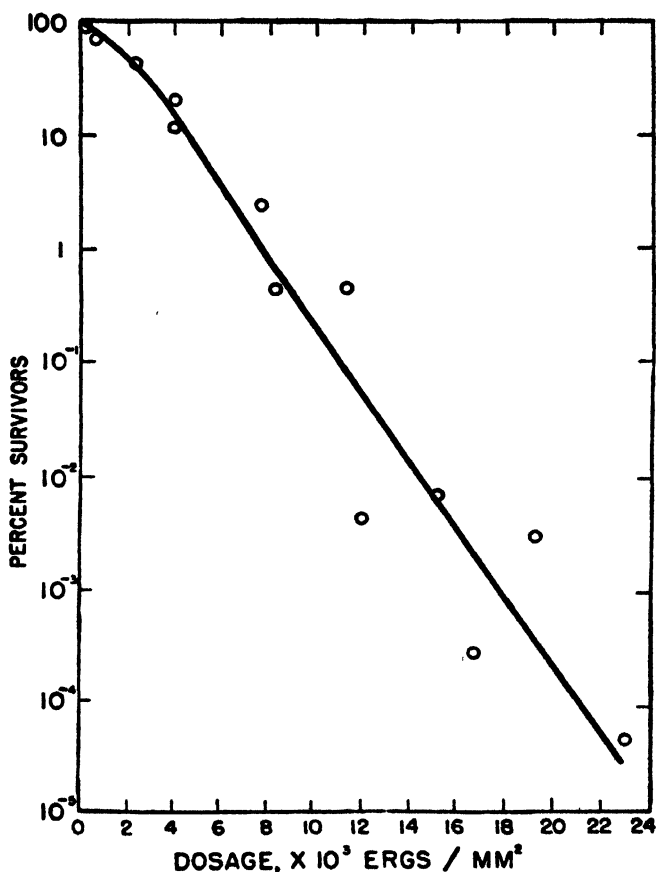
Figure 1. Survival dosage curve of *S. flaveolus* irradiated with ultraviolet light.

There was considerable variability in the mutation frequency in different experiments for suspensions irradiated with the same dose. (Similar variability was found by Hollaender *et al.*, 1945, in *Neurospora.*) For this reason the curves are not averaged, but shown individually in order to determine whether an overall dose mutation frequency pattern was present. Since we were particularly interested in the dosage necessary for maximum mutation frequency, the higher doses were most intensively studied.

The general pattern shown by the curves is for the mutation frequency to increase with dose up to the highest dosage used. There is no evidence that at high

doses the frequency drops, as was reported for various fungi (Hollaender and Emmons, 1941; Hollaender *et al.*, 1945). The highest mutation frequency observed was 24.6 per cent in one experiment at 12,000 ergs $\times$ mm^{-2}. The survival here was 4.2×10^{-5}.

Experiments with X-rays. For the range of X-ray doses studied, 0 to 300,000 roentgens, the survival curve (figure 3) is generally exponential, except for the
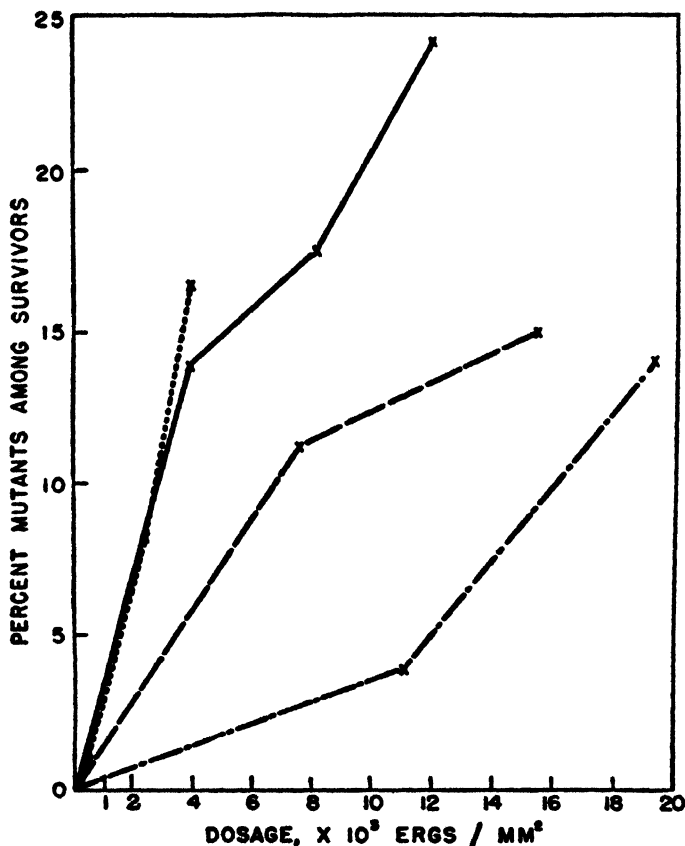
Figure 2. Relation of mutation frequency in survivors to dosage, in ultraviolet-irradiated *S. flaveolus.* The four curves shown represent four independent experiments.

first part, where the rate of killing is higher than at the end of the curve. No data for doses between 0 and 25,000 r were determined.

Figure 4 shows the effect of X-ray dose on mutation frequency. The general pattern shown by the curves is for the frequency to rise almost linearly with dose until about 200,000 r. Above 200,000 r, it falls off or only rises slightly. The highest mutation frequency observed was 15 per cent in one experiment at 300,000 r (survival 1.3×10^{-3}). X-ray experiments were far more uniform than were experiments with ultraviolet.

Most workers have found that with X-rays mutation frequency increased linearly with dose, up to the maximum dosage employed, usually under 100,000 r (e.g., Sansome *et al.*, 1945; Demerec and Latarjet, 1946). Up to 200,000 r our curves, though variable, do not significantly contradict the linear increase theory; obviously, however, there is a leveling off above this dosage. As an additional check on the validity of the curves in figure 4, mutation frequency, X-ray dosage curves were prepared by scoring as mutants *all* strains differing in any observable morphological manner from the wild type. Although this method had the disadvantage of possibly including nonmutant types, it was likely that if the mutation
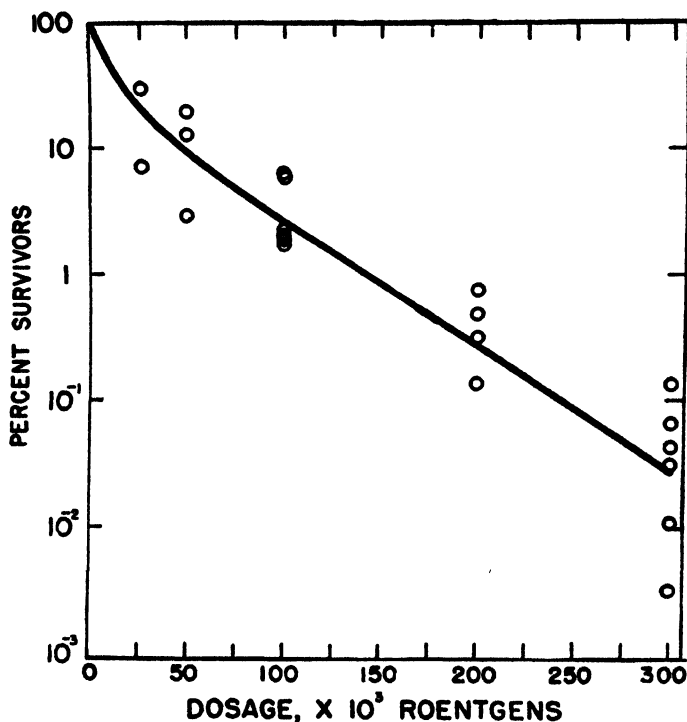
Figure 3. Survival dosage curve of *S. flaveolus* irradiated with X-rays.

rate was increasing significantly above 200,000 r, such curves would show this increase. Results were irregular, but showed in most cases a leveling off above 100,000 r. There was consistently, however, a definite rise in the curve up to the highest dose tested, 300,000 r, contrary to the curves in figure 4 in which only the specific mutants, *yellow, asporogenous,* and *restricted,* were scored.

From this data it is clear that the mutation curve does level off above 100,000 to 200,000 r, but whether there is a continued slow rise above this dosage, or no further increase, is not certain and depends on the method of scoring. As Roepke and Mercer (1947) have shown, factors may arise on high dosage irradiation that can change the apparent frequency of mutation. Another partial explanation

of the falling off of the mutation rate is that, at high doses, double mutants (Stubbe, 1937) are formed which are counted as one mutant.

Stability of the mutant types. The stability of several strains each of *yellow*, *asporogenous*, and *restricted* was tested by culturing the first two types on nutrient agar for about 6 weeks at 28 C, during which period the strains were transferred 5 times. The *restricted* mutants were cultured similarly on potato extract tryp-
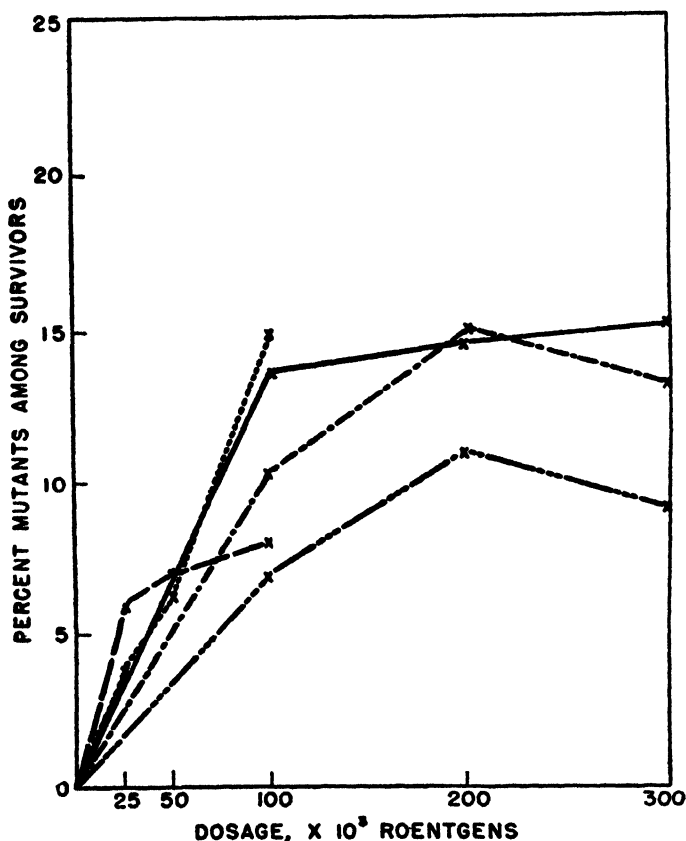
Figure 4. Relation of mutation frequency in survivors to dosage in X-irradiated *S. flaveolus*. The five curves shown represent five independent experiments.

tone medium, M-130 (Kelner and Morton, 1947). After these transfers the strains were seeded again to asparagine glucose agar and scored for the retention of mutant characteristics.

Out of 10 yellow mutants, 3 reverted completely to the wild type, 4 retained yellow pigment to a lesser degree than the original, and 3 remained stable. Two of the latter remained perfectly stable subsequently for over 5 more transfers. Of 5 asporogenous mutants, 2 reverted completely to the sporogenous wild type, 1 sporulated sparsely, and 2 remained stable. Of 5 restricted mutants, 1 re-

verted to the wild form, 2 changed to an intermediate form, and 2 remained stable.

The types of mutants observed in this study suggest that a study of induced mutants may help clear up many perplexing problems in actinomycete taxonomy. Of particular interest is the fact that the *yellow* mutant answered the description for *S. flaveolus* in *Bergey's Manual* (1939) and the original description of the species (*Actinomyces* 168 in Waksman, 1919) far more accurately than did the *S. flaveolus* culture as received from the American Type Culture Collection. The well-known departure of actinomycete cultures from printed description has made identification and classification of individual isolates a most difficult task. In the present case, had not the culture borne its name on the label, it would have been difficult (although not impossible) to identify it as *S. flaveolus.*[6] The yellow mutants induced by irradiation, however, instantly made the relationship clear.

If mutations to types resembling ancestral forms can indeed be readily induced as suggested by our experiments (see also Giles and Lederberg, 1948), then there becomes available a means for possibly determining the ancestral strain of a culture suspected of having undergone variation.

A study of induced mutants also would disclose unsuspected relationships, e.g., the close relation of *S. flaveolus* with asporogenous actinomycetes, or with actinomycetes unable to grow on asparagine glucose agar. A study of the induced (or spontaneous) mutants of a culture along with the usual cytological and physiological studies could furnish bases for taxonomic differentiation of actinomycete groups—each group consisting of strains having among other qualities common types of induced mutants.

The ultraviolet mutation frequency dosage curve differed from the usual type in not showing a fall with maximum doses; however it resembled the "end point" mutation frequency dosage curve for ultraviolet-irradiated *Escherichia coli* (Demerec and Latarjet, 1946). By "end point" mutation is meant a mutation that becomes phenotypically apparent only after one or more cell divisions. In both curves, mutation frequency rises with dosage up to the maximum dosage used.

Since the experiments with X-rays were more reproducible, X-rays appear to be preferable to ultraviolet as a tool for inducing mutations in *S. flaveolus*. In one experiment with ultraviolet, however, a mutation frequency, 24 per cent, higher than that found in any of the X-ray experiments was observed.

SUMMARY

A representative actinomycete species, *Streptomyces flaveolus* ATCC 3319, was irradiated with ultraviolet light and with X-rays in order to determine quantitatively the effect of these agents on the mutation frequency of this organism.

[6] On asparagine glucose agar with concentrations of glucose higher than usual (12.5 g per l instead of 10.0 g per l) the wild strain did sometimes have a fleeting, very pale yellow pigmentation.

Mutation in gross morphology was the mutation type scored. The most common types of mutants were *yellow*, a pigmented mutant resembling Bergey's description of *S. flaveolus* more closely than the ATCC culture, *asporogenous*, in which aerial spores were absent, and *restricted*, strains growing poorly on asparagine glucose agar.

Ultraviolet irradiation doses up to 20,000 ergs $\times$ mm^{-2} were studied. Results were variable, but in all cases mutation frequency rose steadily with dosage, with no evidence of a falling off of the rate of increase at large doses. The maximum mutation frequency observed was 24 per cent in one experiment.

Doses of X-rays up to 300,000 r were studied. Mutation frequency rose with dosage up until 200,000 r, above which the rate of increase leveled off. The highest mutation frequency observed was 15 per cent.

Since results with X-rays were more uniform than with ultraviolet, X-rays are considered preferable to ultraviolet for inducing mutation in actinomycetes.

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STREPTOMYCES VENEZUELAE, N. SP., THE SOURCE OF CHLOROMYCETIN

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The *Streptomyces* that produces "chloromycetin" differs from those described in *Bergey's Manual* (Breed *et al.*, 1948) and is therefore believed to be a new species for which the name *Streptomyces venezuelae* is proposed.

Two cultures have been studied, the first one isolated at New Haven (Burkholder no. A65) from a soil sample collected in a mulched field near Caracas, Venezuela, the second one isolated at Urbana (Gottlieb no. 8-44) from a compost soil on the horticultural farm of the Illinois Agricultural Experiment Station at Urbana (Ehrlich *et al.*, 1947; Carter *et al.*, 1948; Gottlieb *et al.*, 1948; Smith *et al.*, 1948). The first of these, which we regard as the type culture, has been placed in the Culture Bureau of Parke, Davis and Company at Detroit as no. 04745. The description of morphology is based on the type culture but both cultures were employed in the physiologic tests.

MORPHOLOGY

Primary mycelium growing in agar substrata is thin-walled, colorless, hyaline, monopodially branched (figure 1: C). Mature vegetative hyphae vary in diameter from 0.9 to 1.8 microns and the branches grow to about 150 microns in length. Sometimes the substratal mycelium forms oval spores by fragmentation (figure 1: B). The aerial mycelium is lavender under the microscope, thick-walled, generally not much branched, straight or slightly and irregularly curved, not forming loops or spirals, having individual filaments that appear stiff, and arising frequently from the primary mycelium at the surface of the substrate (figure 1: H, I, J). Individual filaments are rarely or not septate, 1.0 to 1.8 microns in diameter, and vary in length up to about 350 microns. In young colonies, the stiff aerial hyphae project outward radially over the surface of the colony and show a lavender color when examined microscopically. The color of colonies when viewed on agar without magnification is gray to light tan or pink, but not lavender. Distal portions of the aerial hyphae commonly subdivide into unbranched oidial spore chains (figure 1: A) which are readily fragmented into small groups or individual spores.

The spores are oval to oblong. Mature spores range from about 0.4 to 0.8 microns in diameter and from 0.7 to 1.6 microns in length. The spores formed by fragmentation of hyphae in the substrate are generally smaller than those

formed from the aerial hyphae. Individual spores are colorless at maturity but in mass appear tan to gray when viewed without magnification. They may be stained readily with crystal violet and other bacteriological dyes. The
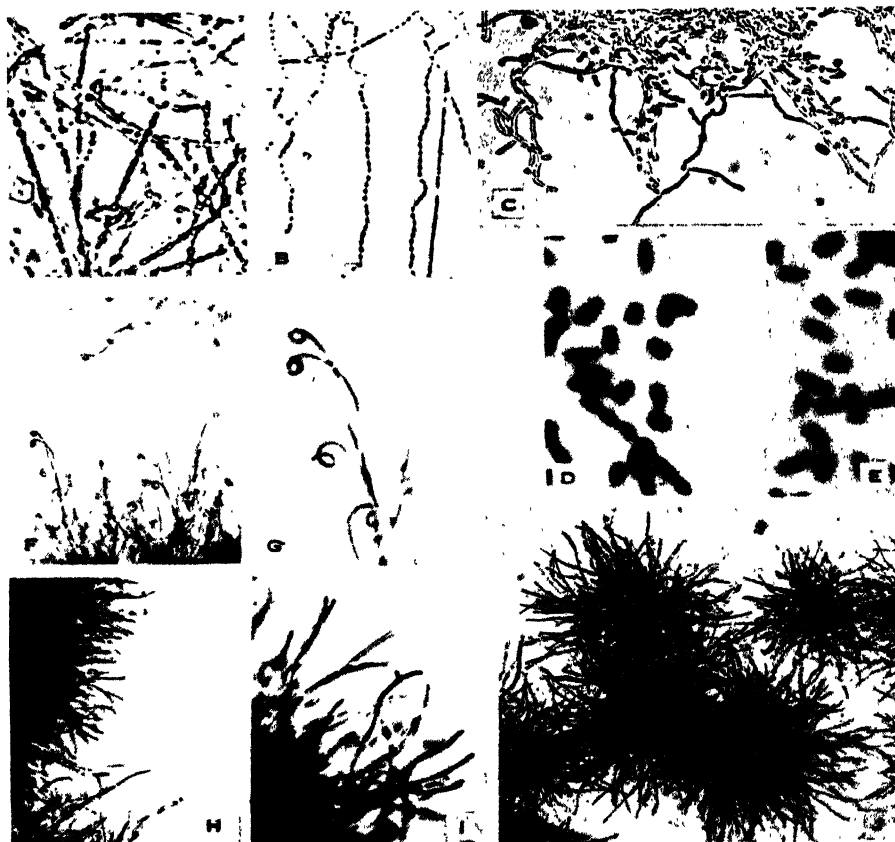
Figure 1. Streptomyces venezuelae: A, aerial hyphae fragmented into chains of spores B, substratal hyphae forming spores in chains; C, young primary mycelium developing many short branches; D, E, spores stained with Giemsa to show the nuclei and lightly counterstained with safranine Y; H, I, aerial hyphae about ready to form spores; J, young colonies forming stiff lavender aerial hyphae. *S. lavendulae:* F, G, aerial hyphae showing characteristic spirals.

Photographs A, B, C, G, I taken with 4-mm dry objective and × 10 ocular using living material growing on agar-covered glass microscope cover slips (magnification × 450). Photographs F, H, and J taken from living cultures on agar with 16-mm objective and × 10 ocular (magnification × 150). Photographs D and E taken with 1.8 mm oil immersion objective and × 10 ocular (magnification, × 3,000) using Giemsa-stained material mounted in "permount."

spores are uninucleate, as determined by Giemsa staining[4] (figure 1: D, E). Mycelium and spores are gram-positive.

[4] A spore suspension in distilled water is fixed on a slide by inverting a hanging droplet over 2 per cent osmic acid solution for 5 minutes and drying in air. After washing for 10 minutes in water, the slide is stained in Giemsa solution phosphate buffer pH 7.4, using 1

Spores of *Streptomyces venezuelae* were sown on various media and incubated at room temperature or at 28 C in order to establish a spectrum of nutritional sources and of effects on certain differential media. The results are compiled in table 1.

STREPTOMYCES VENEZUELAE VS. STREPTOMYCES LAVENDULAE

Since *Streptomyces venezuelae* resembles *S. lavendulae* in certain respects (Carter *et al.*, 1948), cultures of the two species were compared in a variety of ways in order to obtain data on which to judge whether or not they might reasonably be regarded as separate species.

Morphology

Several conspicuous differences in morphology between cultures of *Streptomyces venezuelae* and *S. lavendulae* are apparent upon inspection of the characteristics shown in table 2.

Physiology

Nutritional spectrum and sporulation. Cultures of Waksman's strain 8 of *S. lavendulae* were compared with both cultures of *S. venezuelae* in the nutrition tests reported above. Although the three cultures gave essentially similar results in the majority of the test media, the two *S. venezuelae* strains differed from *S. lavendulae* in their ability to utilize a number of carbohydrates. These differences are shown in table 3 (A). The two species differed also in their requirements for sporulation, as shown in table 3 (B).

Antibiotic production. In order to determine whether *Streptomyces venezuelae* differs from *S. lavendulae* in the nature of the antibiotics produced in different media, the two cultures of *S. venezuelae* and the Parke, Davis subculture of Waksman's strain 8 *S. lavendulae* were grown in shaken flasks in media known to favor biosynthesis of chloromycetin and in others favorable to streptothricin formation. The results of one of several such tests are shown in table 4 and figure 2.[5]

When the flask cultures were assayed for streptothricin content against two test bacteria known to be sensitive to streptothricin but relatively insensitive to chloromycetin (table 4), the two strains of *S. venezuelae* were seen to have produced antibacterial material equivalent to from < 1 to 5.5 streptothricin units per ml in all three media, whereas *S. lavendulae* had produced close to 100 streptothricin units in a streptothricin medium, approximately 15 units in one chlor-

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ml of dye solution (0.5 g National Aniline Division powder in 33 ml glycerol plus 33 ml methyl alcohol) in 30 ml buffer. The slide is allowed to stain until a colored spot becomes visible on the slide (about 15 minutes), then it is decolorized in acetone, counterstained in aqueous safranine, and dehydrated up through a series of acetone-xylol mixtures until in pure xylol. The preparation is mounted in "permount" or other similar material. The nuclei stain blue and the rest of the cell is red.—P.R.B.

[5] Robert M. Smith of Parke, Davis and Company grew the shaken cultures.

TABLE 1
Response of S. venezuelae to various media

A. Miscellaneous

SUBSTRATE	DARKENING	OTHER EFFECTS
Gelatin.......	+	Liquefaction
Litmus milk.....	+	Peptonization, basic reaction
Nitrate broth....	+	Reduction to nitrite
Kligler iron agar..................... ..	+	H$_2$S production
Tryptone broth....	+	No indole production
Dorset egg agar.......	+	—
Potato plug.....	+	—
Tyrosine broth......	+	—
Glucose nutrient agar...	+	—

B. Nitrogen sources
Medium: Synthetic agar* + 1% starch + N source at conc. of 0.106 g N per liter

N SOURCE	GROWTH
Ammonium sulfate.	+
Sodium nitrate...	+
Sodium nitrite..	+†
Acetamide.....	Slight
Asparagine....	+
L-Tyrosine.....	+
DL-Tryptophan.....................................	+

C. Carbohydrate sources
Medium: Synthetic agar* + 0.264% (NH$_4$)$_2$SO$_4$ + CHO source at concentrations indicated below

CHO SOURCE	CONCENTRATION	GROWTH
	per cent	
Pentoses......	1.0	
Arabinose...		+
Rhamnose...		+
Ribose..		Slight
Xylose....... -		+
Hexoses...	1.0	
Glucose...		+
Galactose...		+
Fructose.....		+
Mannose......		+

* KH$_2$PO$_4$.................2.38   g ,   MnCl$_2$·4H$_2$O...................... 0.0079 g
  K$_2$HPO$_4$.................5.65   "   ZnSO$_4$·7H$_2$O...................... 0.0015 "
  MgSO$_4$·7H$_2$O..............1.00   "   Difco agar........................15.0   "
  CuSO$_4$·5H$_2$O...... ....0.0064 "   Distilled water.................... 1 liter
  FeSO$_4$·7H$_2$O...............0.0011 "   Medium adjusted to pH 6.8–7.0

† The cultures failed to grow in the presence of sodium nitrite at a concentration of 2.64 grams per liter.

TABLE 1—*Continued*

CHO SOURCE	CONCENTRATION	GROWTH
	per cent	
Disaccharides	1.0	
Cellobiose		+
Lactose		+
Maltose		+
Sucrose		Slight
Polysaccharides	1.0	
Dextrin		+
Inulin		Slight
Raffinose		Slight
Starch		+
Polyhydric alcohols	1.0	
Dulcitol		Slight
Erythritol		Slight
Glycerol		+
Inositol		Slight
Mannitol		−
Sorbitol		Slight
Sodium salts of organic acids	0.15	
Acetate		+
Citrate		+
Formate		−
Malate		Slight
Oxalate		−
Salicylate		−
Succinate		+
Tartrate		−
Miscellaneous		
o-Cresol	0.1	−
m-Cresol	0.1	−
p-Cresol	0.1	−
Phenol	0.1	−
Salicin	1.0	+

TABLE 2

Some morphological characteristics of S. venezuelae and S. lavendulae

CHARACTERISTIC	S. VENEZUELAE[*]	S. LAVENDULAE[†]
Colony color before sporulation	Gray to light tan or pink	Lavender
Spore color in mass	Tan to gray	Pink to lavender
Aerial hyphae	Stiff and straight or slightly curved (Fig. 1: J, H, I)	Markedly curved or spirals (Fig. 1: F, G)
Spore size in microns	0.7–1.6 × 0.4–0.9	1.6–2.0 × 1.0–1.2

[*] P.D. 04745 and Ill. 8-44.
[†] Waksman 8.

omycetin medium, and only 5 in another. Both *S. venezuelae* cultures differed from *S. lavendulae* also in their relative activity against *Bacillus subtilis* and *Es-*

cherichia coli, their coli:subtilis ratios being 3.3 to 4.3 and 0.7 to 1.2, respectively, in all three media.

When the flask culture filtrates were tested for antibacterial titer and assayed for chloromycetin content against a test bacterium known to be sensitive to chloromycetin (figure 2), the two *S. venezuelae* cultures were seen to have produced similar amounts of antibacterial material in all three media, whereas *S. lavendulae* had produced little measurable activity in the streptothricin medium and less in the two chloromycetin media. One of the two cultures of *S. vene-*

TABLE 3

Some physiological characteristics of S. venezuelae and S. lavendulae

CULTURE MEDIUM	S. VENEZUELAE	S. LAVENDULAE
A. Carbohydrate utilization		
Pentoses		
Arabinose	+	? to −
Rhamnose	+	? to −
Ribose	Slight	−
Xylose	+	? to −
Others		
Lactose	+	Slight
Fructose	+	?
Sodium acetate	+	+ to −
B. Sporulation		
Moyer's penicillium sporulation agar	+	± to −
Synthetic agar (table 1)		
+ glucose and asparagine	± to −	+
+ B vitamins	± to −	+
+ casein hydrolyzate	± to −	+
+ yeast extract	± to −	+
+ tyrosine	± to −	+
Yeast-beef agar	± to −	+
Glucose-tryptone agar	± to −	+

+ = positive; ± = sparse; − = negative; ? = doubtful.

zuelae was slightly more productive than the other in all three media. The two cultures did not vary in their response to the different media: both gave lower yields in medium B than in A and, oddly, both were most productive in C, the streptothricin medium.

Thus, *S. lavendulae* in a streptothricin medium showed high activity by a streptothricin assay and low activity by a chloromycetin assay, whereas in two chloromycetin media it showed low activity by a streptothricin assay and none by a chloromycetin assay. *S. venezuelae*, on the other hand, showed low activity in all three media by a streptothricin assay and relatively high activity in all media by a chloromycetin assay.

In order to compare the antibiotic substances produced by the three strains, the shaken flask cultures of *S. venezuelae* grown in medium A and *S. lavendulae* grown in medium C (table 4, figure 2) were fractionated[6] as shown in figure 3.

TABLE 4

Antibacterial activity (streptothricin units per ml) vs E. coli and B. subtilis of S. venezuelae and S. lavendulae in three shaken culture media

CULTURE MEDIUM FAVORABLE FOR	ASSAY SPECIES USED	ANTIBACTERIAL ACTIVITY OF SHAKEN FLASK CULTURES OF					
		S. venezuelae				S. lavendulae	
		P.D. 04745		Ill. 8-44		Waksman 8	
		Potency	Ratio	Potency	Ratio	Potency	Ratio
		u/ml*	E.c./B.s.	u/ml*	E.c./B.s.	u/ml*	E.c./B.S.
Chloromycetin							
A	E. coli	5.5	4.2	4.3	>4.3	16	1.1
	B. subt.	1.3		<1.0		14	
B	E. coli	4.1	>4.1	3.3	>3.3	5.8	1.2
	B. subt.	<1.0		<1.0		4.7	
Streptothricin	E. coli	3.7	>3.7	3.9	>3.9	74	0.7
C	B. subt.	<1.0		<1.0		105	

Composition of media (percentages)

	A	B	C
Carbo-hydrate	Glycerol.... ...... 1.0	Cerelose... . ... 1.0	Maltose.......... 2.0
Nitrogen	Hog stomach residue 0.5	Soy bean oil meal... 1.0	Corn steep liquor. 6.0
Supple-ment	B-Y fermentation solubles (CSC)... 0.5	Curbay BG (USIC). 0.05	
Salt	NaCl.............. 0.5	NaCl.............. 0.5	NaCl.. ... . 0.2
	CaCO₃............. 0.1	CaCO₃...... ..... 0.1	K₂HPO₄.... ..... 0.2

* Potency is expressed as streptothricin units when assayed by the paper disk agar plate method with a standard curve plotted from dilutions of a streptothricin sulfate preparation kindly supplied by Dr. George F. Cartland of The Upjohn Company, Prep. no. 239–WGJ–4, assumed to contain 400 streptothricin units per milligram. Each potency figure in the table represents the mean of triplicate flasks shaken for 5 days at 22 to 24 C. These assays were performed by Mrs. Frances E. Guest, Dorothy E. Kohberger, and Blanche M. Duckworth of Parke, Davis and Company.

Because chloromycetin is more soluble in ethyl acetate than in water and strepto-thricin is relatively insoluble in ethyl acetate, it was reasoned that assayable activity in the aqueous layers could be streptothricin, whereas that in the solvent-extracted residues could be regarded as chloromycetin but not streptothricin.

[6] Dr. Quentin R. Bartz of Parke, Davis and Company directed the fractionations.

The experimental data compiled in table 5 lead inescapably to the conclusion that the two strains of *S. venezuelae* produced no streptothricin and that *S. lavendulae* produced no chloromycetin. The aqueous layers from the solvent-extracted *S. venezuelae* filtrates exhibited practically no activity against *B. subtilis*, whereas the solvent-extracted residues contained all or nearly all of the activity of the filtrates when assayed against *Shigella*. In contrast, the aqueous layer
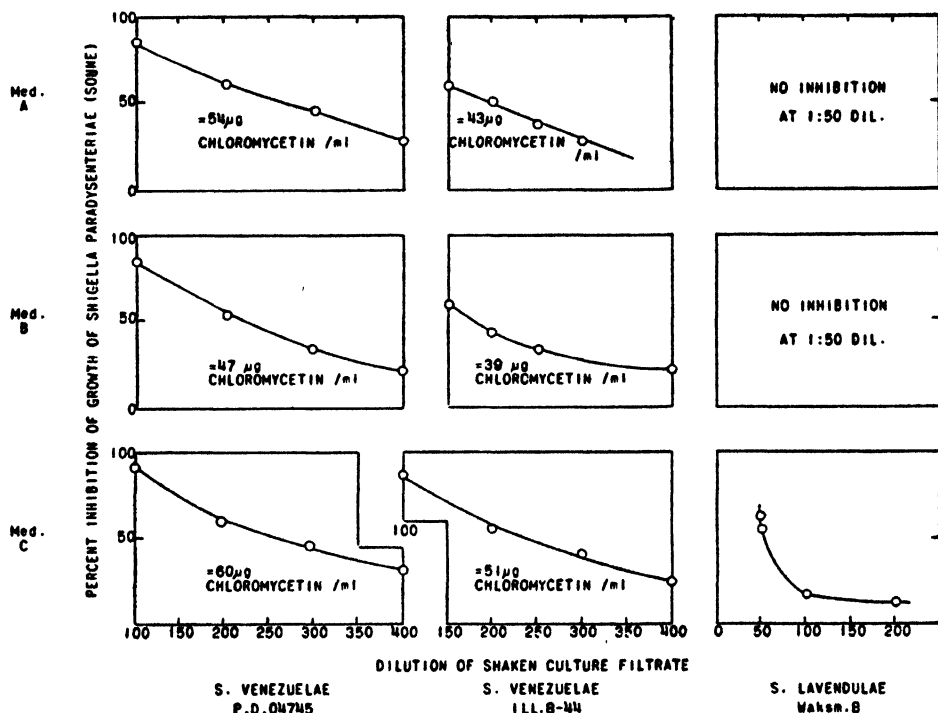
Figure 2. Antibacterial activity (per cent of inhibition over dilution, and equivalent micrograms chloromycetin per ml) vs. *Shigella paradysenteriae* (Sonne) of *S. venezuelae* and *S. lavendulae* in three shaken culture media.

These assays were made on Seitz filtrates of the same shaken flask cultures as those in table 4, but after 6 days. They were performed by a turbidimetric broth dilution method, employing a crystalline chloromycetin standard for the gravimetric estimates of potency (Joslyn and Galbraith, 1947; Smith *et al.*, 1948). The assays were run by Dwight A. Joslyn and Mrs. Margaret Galbraith of Parke, Davis and Company.

from the *S. lavendulae* filtrate exhibited considerable activity against *B. subtilis*, but the solvent-extracted residue showed practically none. Also noteworthy is the relative stability of the *S. venezuelae* culture filtrates at pH 9.5 and 100 C (characteristic of chloromycetin but not of streptothricin) in contrast to the expected instability of the *S. lavendulae* filtrate under these conditions.

Susceptibility to actinophages. The appearance of an actinophage in a subculture of the P.D. 04745 strain of *S. venezuelae* provided the opportunity to ascertain whether or not the available strains of *S. venezuelae, Streptomyces*

griseus, and *S. lavendulae* were all susceptible to the same actinophages. The two strains of *S. venezuelae* and two strains of *S. lavendulae* were seeded on the surfaces of agar plates and streaked with Seitz filtrates of actinophage-containing shaken cultures of the P.D. strain of *S. venezuelae* and two strains of *S. griseus*. Actinophages were not available from the Ill. 8-44 strain of *S. venezuelae* or from *S. lavendulae*. Plates seeded with *S. griseus* strains were also included in order to check on the susceptibility of these strains to the available actinophage-containing filtrates. Table 6 shows the results of these experiments. Both strains
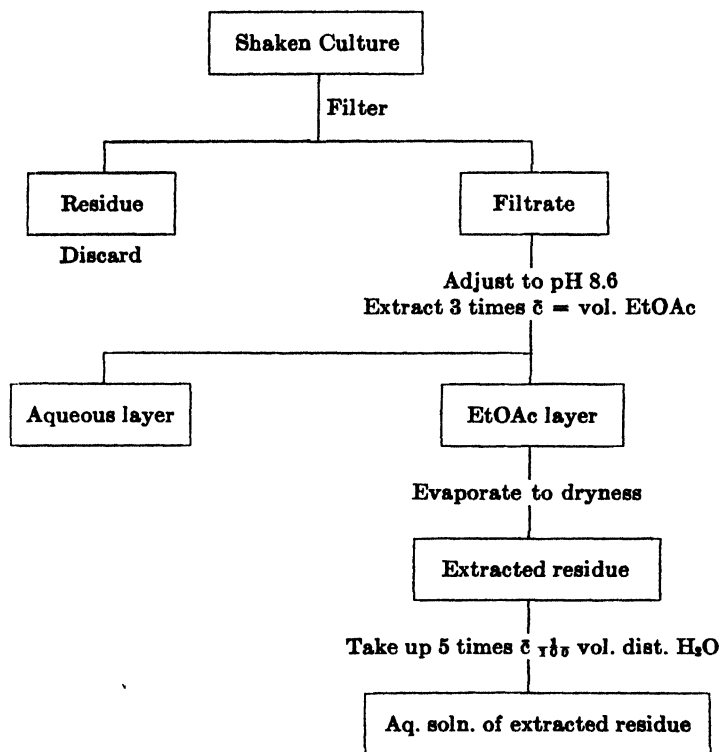
Figure 3. Fractionation of shaken cultures of *S. venezuelae* in medium A and *S. lavendulae* in medium C.

of *S. venezuelae* but neither of the two strains of *S. lavendulae* were lysed by the actinophage from P.D. 04745, thus contributing another point of difference between the two species. The four *S. griseus* strains were unaffected by the *S. venezulae* phage but all—insofar as tested—were susceptible to at least one of the *S. griseus* phages. Excepting one unexplained result, no strain of *S. venezuelae* or *S. lavendulae* was susceptible to any of the *S. griseus* phages against which it was tested. It may be noted that these limited data constitute examples of specificity among these actinophages; that is, each of these phages proved able to lyse all the tested antibiotic-producing strains of the actinomycete species from

TABLE 5

Antibacterial activity of fractions from shaken cultures of S. venezuelae in medium A and S. lavendulae in medium C

FRACTION	DISK PLATE ASSAY VS. B. SUBTILIS, A.T.C.C. 6633			TURBIDIMETRIC ASSAY VS. SHIGELLA PARADYSEN-TERIAE (SONNE), P.D. 04628				
	S. venezuelae		S. laven-dulae	S. venezuelae		S. laven-dulae	S. venezuelae	
	P.D. 04745	Ill. 8-44	Waksman 8	P.D. 04745	Ill. 8-44	Waksman 8	P.D. 04745	Ill. 8-44
	streptothricin units/ ml			*dilution causing 50% inhib.*			*μg chloromycetin/ml*	
Culture filtrate............	3	2	72	265	182	36	49	38
Stability tests								
pH *°C* *min*								
2.0 20 30	—	—	72	240	180	—	45	37
2.0 100 15	—	—	72	235	170	—	44	34
9.5 20 30	—	—	76	245	220	24	48	44
9.5 100 15	—	—	15	195	170	<1*	38	34
Aqueous layer......... ...	<1	0	56	—†	—†	—†	—†	—†
Aqueous solution of residue from ethyl-acetate-extracted fraction.........	60	57	<2	4,260	3,760	<1‡	920	770§
Yield: Total activity of residue + total activity of filtrate.....				80%	103%	—	94%	101%

* 1–10 dilution caused only 20 per cent inhibition.

† These values are low and have no significance because the ethyl acetate dissolved in the water possesses activity against *Shigella paradysenteriae* (Sonne) in this test.

‡ 1–10 dilution caused less than 20 per cent inhibition.

§ Estimated without standard curve for day tested.

TABLE 6

Susceptibility of strains of S. venezuelae, S. griseus, and S. lavendulae to actinophage-containing culture filtrates of some of these strains*

	LYSIS OF AGAR PLATE SEEDINGS OF							
ACTINOPHAGE CONTAINING CULTURE FILTRATES OF	S. venezuelae		S. lavendulae		S. griseus			
	P.D. 04745	Ill. 8-44	W.† 8	W. 14	W. 4	W. 9	W. 10	W. 19
S. venezuelae								
P.D. 04745.......	+	sl. +	0	0	0	0	0	0
S. griseus								
W. 4 (Upjohn subcult.)..	—	0‡	—	—	+	—	—	—
W. 9 (P.D. ")..	0	0	0	0	+	+	+	+
W. 9 (Lilly ")..	—	0§	—	0§	—	+§	—	—

+ = lysis.   0 = no lysis.   — = not tested.

* Except as otherwise noted, these tests were made by Robert M. Smith of Parke, Davis and Company.

† W. = Waksman.

‡ Gottlieb's result; but Colingsworth (Gottlieb *et al.*, 1948) obtained lysis.

§ Lilly results, kindly communicated by Dr. J. M. McGuire of the Lilly Research Laboratories.

which it had been isolated, but none of the tested strains of other actinomycete species.

Serological comparison of cultures.[7] Rabbits were immunized for 3.5 months with weekly intravenous injections of saline suspensions of living mycelium and spores of the cultures. The sera were then tested for precipitins against antigens prepared as saline extracts of the fungi. Strong precipitation occurred between the sera and the homologous antigens of each strain and between the sera and heterologous antigens of the *S. venezuelae* strains. Weak reactions occurred between the sera and heterologous antigens of *S. lavendulae* and the *S. venezuelae* strains.

The demonstration of strong cross reactions between the *S. venezuelae* strains is interpreted as further evidence of their specific identity, and the absence of cross reactions between either of them and *S. lavendulae* as further evidence that they are specifically distinct from *S. lavendulae*.

SUMMARY

The actinomycete that produces "chloromycetin" is described as a new species for which the name *Streptomyces venezuelae* is proposed.

The decision to regard *S. venezuelae* as a species distinct from the somewhat similar *Streptomyces lavendulae* is based on differences in morphology, nutrition, antibiotic production, susceptibility to actinophages, and serological reactions.

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[7] These tests were performed in the Research Laboratories of Parke, Davis and Company by Dr. A. B. Hillegas and Miss Marion McCracken, who will report their work in detail at a later date.

THE INFLUENCE OF SUBCULTURE MEDIA ON RESULTS OBTAINED IN DISINFECTANT TESTING

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It has been known for a long time that some disinfectants have such great bacteriostatic activity that slight traces of the disinfectant carried over into ordinary subculture media prevent the growth of test organisms without killing them. One way of overcoming this difficulty has been to add a neutralizing agent to the subculture media as, for example, ammonium chloride is added when formaldehyde is being tested. However, with phenol and many other disinfectants it has been assumed that simple dilution was sufficient to eliminate the bacteriostatic effects of the small amount of disinfectant that may be carried over.

This commonly accepted assumption has been challenged by Flett and his associates (1945). They report that by adding activated charcoal or ferric chloride to the broth used in determining phenol coefficients by the FDA method, it was possible to secure growth after exposure to dilutions of phenol which appeared to kill the test organisms when subcultures were made into the regular FDA broth.

In attempting to duplicate the results reported by Flett and his associates, a number of comparative tests were made with *Staphylococcus aureus* and *Eberthella typhosa* as the test organisms, grown in FDA broth. A series of experiments were carried out with ferric chloride and a second series with activated charcoal. In each experiment of the first series parallel subcultures were made in FDA broth with and without the addition of ferric chloride. The ferric chloride stock solution was a 1 per cent solution of anhydrous ferric chloride. Two different samples of ferric chloride were used, and one of the stock solutions was subjected to chemical analysis, which showed that the solution contained chlorine and iron in the proper amounts and proportions for a 1 per cent solution. The results obtained are shown in table 1. With both test organisms the addition of ferric chloride to the subculture medium made no difference in the results. When *Staphylococcus aureus* was the test organism and the amount of ferric chloride added was 0.1 per cent, there was less growth, due undoubtedly to acidity of the culture medium. Similar experiments were also conducted in which subcultures were made into beef extract broth containing 0.1 per cent of "norit." In these experiments, also, no appreciable amount of increased growth was noted. In order to ascertain whether other factors might influence the results, a series of experiments were then conducted in which some subcultures were made in beef extract broth and some in beef infusion broth. Each of these subculture media contained 1 per cent of Armour's peptone, and they were adjusted to approxi-

TABLE 1

Effect of addition of ferric chloride to subculture medium

DISINFECTANT	DILU-TION	FeCl₃	EXPOSURE TIME (in minutes)						TEST ORGANISM
			2.5	5.0	7.5	10.0	12.5	15.0	
Experiment 1									
Phenol	1–50	% None	−	−	−	−	−	−	*Staphylococcus aureus* grown in beef extract broth
	1–55		+	−	−	−	−	−	
	1–60		+	+	+	−	−	−	
	1–65		+	+	+	+	+	+	
	1–50	0.03	−	−	−	−	−	−	
	1–55		+	−	−	−	−	−	
	1–60		+	+	+	−	−	−	
	1–65		+	+	+	+	+	+	
	1–60	None	−	−	−	−	−	−	*Eberthella typhosa* grown in beef extract broth
	1–70		+	−	−	−	−	−	
	1–80		+	+	+	−	−	−	
	1–90		+	+	+	+	+	+	
	1–60	0.03	−	−	−	−	−	−	
	1–70		+	−	−	−	−	−	
	1–80		+	+	+	+	−	−	
	1–90		+	+	+	+	+	+	
Experiment 2									
Phenol	1–55	None	+	−	−	−	−	−	*Staphylococcus aureus* grown in beef extract broth
	1–60		+	+	−	−	−	−	
	1–65–		+	+	+	−	−	−	
	1–70		+	+	+	+	+	+	
	1–55	0.05	+	−	−	−	,−	−	
	1–60		+	+	−	−	−	−	
	1–65		+	+	+	+	−	−	
	1–70		+	+	+	+	+	+	
	1–60	0.1	−	−	−	−	−	−	
	1–65		+	−	−	−	−	−	
	1–70		+	+	+	−	−	−	
	1–70	None	+	−	−	−	−	−	*Eberthella typhosa* grown in beef extract broth
	1–75		+	+	−	−	−	−	
	1–80–		+	+	+	−	−	−	
	1–85		+	+	+	+	+	+	
	1–70	0.05	+	−	−	−	−	−	
	1–75		+	+	+	−	−	−	
	1–80		+	+	+	+	−	−	
	1–70	0.1	+	−	−	−	−	−	
	1–75		+	+	+	−	−	−	
	1–80		+	+	+	+	−	−	

Experiments conducted at 20 C.

mately the same pH.   The results obtained with phenol as the disinfectant are shown in table 2.   There was no difference between the results on the two subculture media when *Staphylococcus aureus* was the test organism, but there was

TABLE 2

Comparative tests with beef extract and beef infusion subculture media

DISIN-FECTANT	DILU-TION	SUBCULTURE MEDIUM	EXPOSURE TIME (IN MINUTES)						TEST ORGANISM
			2.5	5.0	7.5	10.0	12.5	1.50	
Phenol	1-50	Beef extract	−	−	−	−	−	−	*Staphylococcus aureus*
	1-55	broth	+	+	−	−	−	−	grown in beef ex-
	1-60		+	+	+	−	−	−	tract broth
	1-65		+	+	+	+	+	+	
	1-70		+	+	+	+	+	+	
	1-50	Beef infusion	−	−	−	−	−	−	
	1-55	broth	+	−	−	−	−	−	
	1-60		+	+	+	−	−	−	
	1-65		+	+	+	+	+	+	
	1-70		+	+	+	+	+	+	
	1-65	Beef extract	−	−	−	−	−	−	*Eberthella typhosa*
	1-70	broth	+	−	−	−	−	−	grown in beef ex-
	1-75		+	+	−	−	−	−	tract broth
	1-80		+	+	+	+	−	−	
	1-85		+	+	+	+	+	+	
	1-60	Beef infusion	+	−	−	−	−	−	
	1-65	broth	+	+	−	−	−	−	
	1-70		+	+	+	+	+	−	
	1-75		+	+	+	+	+	+	
	1-80		+	+	+	+	+	+	
	1-65	Beef extract	−	−	−	−	−	−	*Escherichia coli* grown
	1-70	broth	+	+	−	−	−	−	in beef extract broth
	1-75		+	+	+	+	+	−	
	1-80		+	+	+	+	+	+	
	1-60	Beef infusion	+	−	−	−	−	−	
	1-65	broth	+	+	+	−	−	−	
	1-70		+	+	+	+	+	+	

Experiments conducted at 20 C.

a decided difference when the test organism was *Eberthella typhosa* or *Escherichia coli*.

Similar experiments were conducted with orthocresol, paracresol, liquor cresolis saponatus, parabutylphenol, orthochlorphenol, normal butyl alcohol, and a coal tar emulsion disinfectant.   There was practically no difference, except when orthochlorphenol was the disinfectant, in growth with the two subculture media

when *Staphylococcus aureus* was the test organism. With orthochlorphenol, there was more growth in beef infusion broth than in beef extract broth.

There was practically no difference with *Eberthella typhosa* as the test organism when the disinfectants were liquor cresolis saponatus or coal tar emulsion disinfectant, both of which contained soap. On the other hand, decided differences in results obtained with the two subculture media were noted with *Eberthella typhosa* and *Escherichia coli* as test organisms and orthocresol, paracresol, parabutylphenol, orthochlorphenol, and normal butyl alcohol as the disinfectants.

TABLE 3

Effect of addition of coconut oil soap upon germicidal efficiency of phenols

DISINFECTANT	DILUTION	SUBCULTURE MEDIUM	EXPOSURE TIME (IN MINUTES)						TEST ORGANISM
			2.5	5	7.5	10	12.5	15	
Phenol alone	1-55	Beef extract broth	+	−	−	−	−	−	*Staphylococcus aureus* grown in beef extract broth
	1-60		+	+	+	−	−	−	
	1-65		+	+	+	+	+	−	
	1-70		+	+	+	+	+	+	
Phenol + coconut oil soap = 1/2 phenol	1-75	Beef extract broth	+	−	−	−	−	−	
	1-80		+	−	−	−	−	−	
	1-85		+	+	−	−	−	−	
	1-90		+	+	+	+	−	−	
	1-75	Beef infusion broth	+	−	−	−	−	−	
	1-80		+	+	−	−	−	−	
	1-85		+	+	+	−	−	−	
	1-90		+	+	+	+	−	−	
Phenol alone	1-70	Beef extract broth	+	−	−	−	−	−	*Eberthella typhosa* grown in beef extract
	1-75		+	+	−	−	−	−	
	1-80		+	+	+	+	−	−	
	1-85		+	+	+	+	+	+	
Phenol alone	1-60	Beef infusion broth	+	−	−	−	−	−	
	1-65		+	+	−	−	−	−	
	1-70		+	+	+	+	+	−	
	1-75		+	+	+	+	+	+	
Phenol + coconut oil soap = 1/2 phenol	1-180	Beef extract broth	+	−	−	−	−	−	
	1-200		+	+	−	−	−	−	
	1-220		+	+	+	−	−	−	
	1-240		+	+	+	+	−	−	
	1-180	Beef infusion broth	+	−	−	−	−	−	
	1-200		+	+	−	−	−	−	
	1-220		+	+	+	−	−	−	
	1-240		+	+	+	−	−	−	

TABLE 3—*Continued*

DISINFECTANT	DILUTION	SUBCULTURE MEDIUM	EXPOSURE TIME (IN MINUTES)						TEST ORGANISM
			2.5	5	7.5	10	12.5	15	
Phenol alone	1-65	Beef extract broth	−	−	−	−	−	−	*Escherichia coli* grown in beef extract broth
	1-70		+	+	−	−	−	−	
	1-75		+	+	+	+	+	−	
	1-60	Beef infusion broth	+	−	−	−	−	−	
	1-65		+	+	+	−	−	−	
	1-70		+	+	+	+	+	+	
Phenol + coconut oil soap = 1/2 phenol	1-180	Beef extract broth	+	+	−	−	−	−	
	1-200		+	+	−	−	−	−	
	1-220		+	+	−	−	−	−	
	1-240		+	+	+	+	−	−	
	1-260		+	+	+	+	+	−	
	1-180	Beef infusion broth	+	−	−	−	−	−	
	1-200		+	−	−	−	−	−	
	1-220		+	+	+	−	−	−	
	1-240		+	+	+	−	−	−	
Orthocresol alone	1-100	Beef extract broth	+	−	−	−	−	−	*Staphylococcus aureus* grown in beef extract broth
	1-110		+	+	+	−	−	−	
	1-120		+	+	+	+	+	−	
	1-100	Beef infusion broth	−	−	−	−	−	−	
	1-110		+	+	+	+	−	−	
	1-120		+	+	+	+	+	+	
Orthocresol + coconut oil soap = 1/2 orthocresol	1-100	Beef extract broth	−	−	−	−	−	−	
	1-140		+	+	+	−	−	−	
	1-180		+	+	+	+	+	+	
Orthocresol + soap	1-100	Beef infusion broth	−	−	−	−	−	−	
	1-140		+	+	+	−	−	−	
	1-180		+	+	+	+	+	+	
Orthocresol alone	1-140	Beef extract broth	+	−	−	−	−	−	*Eberthella typhosa* grown in beef extract broth
	1-150		+	+	+	−	−	−	
	1-160		+	+	+	+	+	−	
	1-120	Beef infusion broth	+	−	−	−	−	−	
	1-130		+	+	−	−	−	−	
	1-140		+	+	+	+	−	−	
	1-150		+	+	+	+	+	+	
Orthocresol + coconut oil soap = 1/2 orthocresol	1-280	Beef extract broth	+	−	−	−	−	−	
	1-320		+	+	−	−	−	−	
	1-360		+	+	+	−	−	−	
	1-400		+	+	+	+	−	−	

TABLE 3—*Concluded*

DISINFECTANT	DILUTION	SUBCULTURE MEDIUM	EXPOSURE TIME (IN MINUTES)						TEST ORGANISM
			2.5	5	7.5	10	12.5	15	
	1–280	Beef infusion broth	+	−	−	−	−	−	
	1–320		+	+	−	−	−	−	
	1–360		+	+	+	−	−	−	
	1–400		+	+	+	+	−	−	
Orthocresol alone	1–160	Beef extract broth	+	−	−	−	−	−	*Escherichia coli* grown in beef extract broth
	1–180		+	+	−	−	−	−	
	1–200		+	+	+	+	+	+	
	1–140	Beef infusion broth	+	+	−	−	−	−	
	1–160		+	+	+	+	+	+	
	1–180		+	+	+	+	+	+	
Orthocresol + coconut oil soap = 1/2 orthocresol	1–360	Beef extract broth	+	−	−	−	−	−	
	1–400		+	+	−	−	−	−	
	1–440		+	+	+	+	+	−	
	1–480		+	+	+	+	+	−	
	1–360	Beef infusion broth	+	−	−	−	−	−	
	1–400		+	+	−	−	−	−	
	1–440		+	+	+	−	−	−	
	1–480		+	+	+	+	+	−	

Experiments conducted at 20 C.

The results obtained in experiments with phenol or orthocresol with and without the addition of coconut oil soap are shown in table 3. The amount of soap used was one-half of the amount of phenol or orthocresol used. The addition of coconut oil soap caused a decided increase in bactericidal efficiency against all three test organisms, but less against *Staphylococcus aureus* than against *Eberthella typhosa* or *Escherichia coli*. This increased efficiency was not affected by the use of beef infusion subculture media.

The results obtained in a number of phenol coefficient tests are shown in table 4. The differences between phenol coefficients obtained with beef extract and beef infusion subculture media were less than 10 per cent with all of the disinfectants except the two cresylic disinfectants.

It seemed possible that increased growth in beef infusion subculture media might be due to the presence of growth factors such as those demonstrated by Koser and Saunders (1935) in veal infusion. A comparative experiment was therefore made with phenol as the disinfectant and *Eberthella typhosa* as the test organism with subcultures made (1) into beef extract broth, (2) into beef infusion broth, and (3) into beef infusion broth which had been treated with norit, as suggested by Koser and Saunders. The results obtained with the two beef infusion media were practically identical.

TABLE 4

Results of phenol coefficient tests with beef extract and beef infusion subculture media

DISINFECTANT	DILUTION	SUBCULTURE MEDIUM	EXPOSURE TIME (IN MINUTES)						PHENOL COEFFICIENTS
			2.5	5	7.5	10	12.5	15	
Phenol	1–80	Beef extract broth	+	+	−	−	−	−	
Liquor cresolis saponatus	1–120		+	+	−	−	−	−	
	1–140		+	+	+	+	−	−	120/80 = 1.5
	1–160		+	+	+	+	+	+	
	1–180		+	+	+	+	+	+	
Phenol	1–70	Beef infusion broth	+	+	+	+	−	−	
Liquor cresolis saponatus	1–120		+	+	+	−	−	−	
	1–140		+	+	+	+	+	−	130/70 = 1.85
	1–160		+	+	+	+	+	+	
	1–180		+	+	+	+	+	+	
Phenol	1–80	Beef extract broth	+	+	−	−	−	−	
Cresylic disinfectant no. 3619	1–250		+	−	−	−	−	−	
	1–300		+	+	−	−	−	−	300/80 = 3.75
	1–350		+	+	+	−	−	−	
	1–400		+	+	+	+	+	+	
Phenol	1–65	Beef infusion broth	+	+	−	−	−	−	
Cresylic disinfectant no. 3619	1–250		+	−	−	−	−	−	
	1–300		+	+	−	−	−	−	300/65 = 4.6
	1–350		+	+	+	−	−	−	
	1–400		+	+	+	+	+	−	
Phenol	1–80	Beef extract broth	+	+	−	−	−	−	
Orthocresol	1–160		+	−	−	−	−	−	
	1–170		+	−	−	−	−	−	180/80 = 2.25
	1–180		+	+	−	−	−	−	
	1–190		+	+	+	+	+	−	
Phenol	1–65	Beef infusion broth	+	+	−	−	−	−	
Orthocresol	1–140		+	−	−	−	−	−	
	1–150		+	+	+	−	−	−	145/65 = 2.23
	1–160		+	+	+	+	+	+	
	1–170		+	+	+	+	+	+	

TABLE 4—*Continued*

DISINFECTANT	DILUTION	SUBCULTURE MEDIUM	EXPOSURE TIME (IN MINUTES)						PHENOL COEFFICIENTS
			2.5	5	7.5	10	12.5	15	
Phenol	1–80	Beef extract broth	+	+	–	–	–	–	
Orthochlorphenol	1–220		+	–	–	–	–	–	240/80 = 3.0
	1–240		+	+	–	–	–	–	
	1–260		+	+	+	–	–	–	
	1–280		+	+	+	+	+	+	
Phenol	1–65	Beef infusion broth	+	+	–	–	–	–	
Orthochlorphenol	1–190		+	–	–	–	–	–	200/65 = 3.07
	1–200		+	+	–	–	–	–	
	1–210		+	+	+	–	–	–	
	1–220		+	+	+	+	+	+	
Phenol	1–80	Beef extract broth	+	+	–	–	–	–	
Sodium orthophenyl-phenate	1–1,800		+	+	–	–	–	–	1,800/80 = 22.5
	1–1,900		+	+	+	–	–	–	
	1–2,000		+	+	+	+	–	–	
	1–2,100		+	+	+	+	+	+	
Phenol	1–65	Beef infusion broth	+	+	–	–	–	–	
Sodium orthophenyl-phenate	1–1,500		+	+	–	–	–	–	1,500/65 = 22.3
	1–1,600		+	+	+	+	+	–	
	1–1,700		+	+	+	+	+	+	
	1–1,800		+	+	+	+	+	+	
Phenol	1–80	Beef extract broth	+	+	–	–	–	–	
Coal tar emulsion disinfectant	1–400		+	+	–	–	–	–	400/80 = 5.0
	1–450		+	+	+	–	–	–	
	1–500		+	+	+	+	–	–	
	1–550		+	+	+	+	+	+	
Phenol	1–65	Beef infusion broth	+	+	–	–	–	–	
Coal tar emulsion disinfectant	1–300		+	–	–	–	–	–	350/65 = 5.38
	1–350		+	+	–	–	–	–	
	1–400		+	+	+	+	–	–	
	1–450		+	+	+	+	+	+	

TABLE 4—*Concluded*

DISINFECTANT	DILUTION	SUBCULTURE MEDIUM	EXPOSURE TIME (IN MINUTES)						PHENOL COEFFICIENTS
			2.5	5	7.5	10	12.5	15	
Phenol	1–80	Beef extract broth	+	+	−	−	−	−	
Emulsion disinfectant no. 2	1–900		+	−	−	−	−	−	1,100/80 = 13.7
	1–1,000		+	−	−	−	−	−	
	1–1,100		+	+	−	−	−	−	
	1–1,200		+	+	+	+	+	+	
Phenol	1–65	Beef infu- sion broth	+	+	−	−	−	−	
Emulsion disinfectant no. 2	1–700		+	−	−	−	−	−	900/65 = 13.8
	1–800		+	−	−	−	−	−	
	1–900		+	+	−	−	−	−	
	1–1,000		+	+	+	−	−	−	

Experiments conducted at 20 C.
Test organism *Eberthella typhosa* grown in beef extract broth.

In another experiment beef infusion was boiled, with 2 per cent of norit added to it. After filtration to remove the norit, the filtrate was used for making a culture medium containing the same amounts of peptone and sodium chloride as the regular beef infusion medium and adjusted to the same pH. The results obtained with this subculture medium were the same as those obtained with the regular beef infusion medium. In view of the results obtained in these experiments, it does not seem likely that the presence of growth factors was responsible for the results reported previously in table 2.

SUMMARY

Experiments with phenol as the disinfectant and *Staphylococcus aureus* and *Eberthella typhosa* as test organisms were conducted with the FDA subculture medium with and without the addition of ferric chloride or "norit" to the subculture medium. No significant difference in growth was noted in the medium containing ferric chloride or norit.

Comparative experiments were made with beef extract and beef infusion subculture media, using phenol as the disinfectant and *Staphylococcus aureus, Eberthella typhosa*, and *Escherichia coli* as test organisms. No differences between amounts of growth in the two media were noted when *Staphylococcus aureus* was the test organism, but decidedly greater growth in beef infusion broth was observed when *Eberthella typhosa* and *Escherichia coli* were the test organisms. Similar results were obtained with orthocresol, paracresol, parabutylphenol, orthochlorphenol, and normal butyl alcohol as the disinfectants. Such differences were not observed with disinfectants containing soap.

Phenol coefficient tests with the two subculture media were run with several disinfectants and the differences noted were less than 10 per cent with all of them except two cresylic disinfectants.

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BACTERIOLOGIC STUDIES ON AUREOMYCIN[1]

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Unpublished data made available to us suggested that a new antibiotic, aureomycin,[3] was of low toxicity, was highly effective against a variety of experimental infections in animals, and, therefore, warranted a clinical trial. The bacteriological studies which were conducted as part of the clinical evaluation of this agent are presented in this paper.

SENSITIVITY OF VARIOUS BACTERIA

Aureomycin hydrochloride was found to have considerable antibacterial action *in vitro* against a wide range of gram-positive and gram-negative bacteria. The sensitivities to aureomycin of 186 strains of organisms recently isolated from patients in the Boston City Hospital are presented in table 1. Among the strains of the various organisms tested, only those of *Proteus vulgaris* and of *Pseudomonas aeruginosa* were regularly found to be relatively resistant to aureomycin.

COMPARATIVE SENSITIVITY TO THREE ANTIBIOTICS

The sensitivity to aureomycin, penicillin, and streptomycin of a selected group of microorganisms is presented in table 2. Weight for weight, aureomycin was found to be less effective than penicillin, but more effective than streptomycin, in the case of most coccal organisms. Aureomycin was about as effective as streptomycin against most gram-negative bacilli. Aureomycin possesses equal antibacterial activity against penicillin-sensitive and penicillin-resistant staphylococci as well as against streptomycin-sensitive and streptomycin-resistant bacteria. It is also effective against streptomycin-dependent organisms.

FACTORS INFLUENCING ANTIBACTERIAL ACTIVITY OF AUREOMYCIN

The concentration of antibiotic which is necessary to inhibit any bacterium depends upon many factors, some of which are concerned chiefly with the antibiotic and others of which have largely to do with the microorganisms. Those

TABLE 1

Aureomycin sensitivity of bacteria recently isolated from cases of human infections

ORGANISM	NUMBER OF STRAINS TESTED	INHIBITING CONCENTRATION μG/ML (COMPLETE)
Aerobacter aerogenes............................	10	12.5–50
Diplococcus pneumoniae...	13	0.1–1.0
Escherichia coli.	31	3.1–100*
Hemophilus hemolyticus......	1	0.8
Hemophilus influenzae, type ?.....................	5	1.0–2.0
" " , type b...........	1	2.0
Klebsiella pneumoniae........	4	6.3–50
Neisseria catarrhalis...............	2	2.0
" *gonorrhoeae*........	37	0.25–1.0
" *meningitidis*....	1	0.5
Pleuropneumonialike....	1	0.25
Proteus morganii...............	1	4.0
" *vulgaris*........	13	125–250
Pseudomonas aeruginosa..........	7	100–250
Salmonella, sp.	6	3.1–25
" *typhosa*.... ,..	6	3.1–25
Staphylococcus albus.	2	1.0–2
Staphylococcus aureus...	27	1.0–12.5†
Streptococcus faecalis...	2	6.3
" *mitis*...	4	0.8–6.3
" *pyogenes*....	12	0.5–1.0

Some of these tests were made by a tube dilution method and others by a surface streak method.

* Twenty-two strains inhibited by 12.5 or 25 μg per ml.

† Twenty-four of these inhibited by 1 to 2 μg per ml.

TABLE 2

Sensitivity of selected strains to three antibiotics

ORGANISM	AUREOMYCIN	PENICILLIN	STREPTOMYCIN
D. pneumoniae..................	0.5	0.02	25
E. coli S..............	50	24	6.3
" R......................	50	24	>50,000
" D...........	12.5	24	Dependent
P. morganii S........	50	750	100
" R.........	25	750	>50,000
" D......	12.5	375	Dependent
S. cholerae-suis................	6.3	60	250
S. typhosa......................	6.3	6	100
S. aureus	2	60	25
" S....	6.3	0.08	25
" R..................	6.3	0.08	50,000
" D..............	3.1	0.02	Dependent
S. faecalis......................	6.3	6	3.1
S. mitis......................	1	0.16	6.3
S. pyogenes	0.5	0.02	25

All sensitivities given in μg per ml for complete inhibition.

S, R, and D represent streptomycin-sensitive, -resistant, and -dependent variants, respectively, of the same strain (Paine and Finland, 1948).

which primarily concern the antibiotic may be grouped under two headings: (1) stability of the antibiotic and (2) conditions or substances which inhibit or augment the action of the antibiotic, such as the hydrogen ion concentration and the presence of salts.

The factors which are related particularly to the organisms and which may influence the action of the antibiotic are: (1) the innate susceptibility or resistance of the organism to the antibiotic, (2) the number and growth phase of the microorganisms involved in the test, (3) the presence of conditions or substances which interfere with or promote growth of the organism, such as pH, type of media, etc., (4) the elaboration of substances by the bacteria which may interfere

TABLE 3

Stability of aureomycin in solution

DILUENT	BROTH	H₂O	PLASMA	BROTH	H₂O	BAP	PLASMA
Concentration (μg/ml) of solution stored	8*	2,000†	8*	8*	2,000†	200‡	8*
Temperature C	4	4	4	37	37	37	37
Day 0	0.5§	0.5	0.5	1.0	0.5	1.6	0.5
1	—	—	1.0	4.0	0.5	6.2	2.0
3	0.5	0.5	4.0	>4.0	0.5	12.5	>4.0
7	0.5	0.5			0.5		
14	2.0	0.5			0.5		
21	2.0	1.0			0.5		
28	>4.0	1.0					
35	—	2.0					

* pH 7.2 ±.   †pH 4.

‡ Series of agar plates containing 10 per cent horse blood and graded concentrations of aureomycin from 200 to 0.019 μg per ml.

§ Minimum concentration of aureomycin for complete inhibition of *Streptococcus* no. 98.

with the activity of the antibiotic, and (5) the development of resistance by the microorganism.

Stability of aureomycin: effect of temperature and diluent. Aureomycin, in the form of the crystalline hydrochloride, as made available to us, was a stable preparation and maintained its potency at ±22 C in dried form for at least 6 months. It was also stable in high concentrations in distilled water, both in the refrigerator and in the incubator at 37 C, for periods up to 2 or 3 weeks. The pH of the preparations in distilled water was 4.0 to 4.5. However, when the solutions were kept in low concentrations, either in broth, in pooled human plasma, or incorporated in 10 per cent horse blood agar, there was a rapid loss of activity on incubation at 37 C, and a less rapid loss of activity on refrigeration. Under these circumstances, the pH of the materials was about neutral. The data are shown in table 3.

Natural resistance. Of the organisms studied in this laboratory, only strains of *Proteus vulgaris* and *Pseudomonas aeruginosa* have shown any considerable

degree of innate resistance to aureomycin. These organisms are not completely resistant to aureomycin, however, and can be inhibited *in vitro*, provided that sufficient antibiotic is present. The relative resistance of these two species of gram-negative bacilli may reflect the degree to which these microorganisms will be susceptible to aureomycin therapy, and it might be expected that they would be difficult to eradicate from certain sites where high concentrations of the antibiotic cannot be obtained. This has already been confirmed in preliminary clinical trials.

Effect of inoculum size and growth phase. The number of organisms present was found to influence the concentration of aureomycin required for complete inhibition of bacterial growth in brain heart infusion broth (Difco). However, in sensitivity determinations made by streaking from broth cultures of bacteria directly to the surface of agar plates containing graded concentrations of aureomycin, the number of organisms present in the inoculum had little influence on the concentration of this antibiotic required to inhibit growth. Table 4 shows the influence of the size of the inoculum on inhibition end points obtained with aureomycin when the tests were made by the tube dilution and the surface streak methods with *Streptococcus pyogenes*, strain 98. Similar results were also obtained with a strain of *Klebsiella pneumoniae* and a subtilislike gram-positive bacillus (*Bacillus* no. 5, obtained from Dr. B. M. Duggar).

In the tube dilution method there was little evidence of antibacterial activity when large numbers of organisms were used in the inocula, even in the presence of high concentrations of aureomycin. With fewer organisms in the inocula, the visible end points of growth were not greatly altered in the tube dilution method by changes in the size of the inocula. The surface streak method, however, showed a sharp inhibition end point, which was essentially the same over a wide range of inoculum size. It has been suggested that this difference in inhibition end points observed in the tube dilution and in the surface streak methods, when large inocula are used, may simply reflect the lack of vigorous growth from the large inocula in the tube dilution method, the organisms having already attained nearly their maximum growth with insufficient fresh media provided to allow vigorous multiplication. In the case of the surface streak method, there is not such a limiting factor, so far as the media are concerned, since there probably is vigorous growth both from the large and the small inocula used. These observations suggest that the antibacterial effect of aureomycin is exerted largely on multiplying organisms.

The effect of inoculum size on inhibition of bacterial cultures by aureomycin was also apparent from a study of growth curves, starting with different numbers of bacteria. In figure 1 are shown the growth curves of a beta hemolytic streptococcus in graded concentrations of aureomycin. Progressive inhibition of the rate of growth by increasing concentrations of the antibiotic is apparent and the streptococcus failed to multiply in the presence of 2 μg of aureomycin per ml. In this test a relatively small number of organisms was inoculated and the opportunity for initiating vigorous growth was present. Under these conditions the presence of a relatively small amount of aureomycin, 2 μg per ml, was sufficient to inhibit growth of this organism. On the other hand, when large numbers of

TABLE 4
Effect of size of inoculum on the antibacterial action of aureomycin

INOCU-LUM[*]	METHOD[†]	INCUBA-TION	FINAL CONCENTRATION OF AUREOMYCIN, µG/ML OF MEDIUM									
			100	50	25	12.5	6.3	3.1	1.6	0.8	0.4	0
		hrs										
10⁻¹	A	18	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	B	24	3	+	++	+++	+++	+++	+++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	+++	+++	+++
10⁻²	A	18	0	0	0	0	0	0	0	+	++	+++
	B	24	0	0	0	0	1	+++	+++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	+++	+++	+++
10⁻³	A	18	0	0	0	0	0	0	0	0	+	+++
	B	24	0	0	0	0	0	1	+++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	++	++	++
10⁻⁴	A	18	0	0	0	0	0	0	0	0	+	+++
	B	24	0	0	0	0	0	0	++	+++	+++	+++
	C	24	0	0	0	0	0	0	0	+	+	+

Organism: *S. pyogenes*, no. 98. Media: beef heart infusion broth (Difco), pH 7.2 ±, and heart infusion agar (Difco) to which 10 per cent defibrinated horse blood has been added.

+, ++, and +++ = estimation of growth; 0 = no visible growth; numerals indicate number of discrete colonies observed.

When the inoculum was an undiluted culture (1:2 final dilution in A) there was vigorous growth in all dilutions in A and B and the results in C were identical to those obtained with the 10⁻¹ inoculum.

* Dilution of fully grown culture: 0.5 ml added to each tube containing 0.5 ml of diluted aureomycin in broth; and a 2-mm loopful streaked on the surface of the blood agar plates containing antibiotic.

† A = tube dilution method, reading visible growth; B = subcultures from A to blood agar plates without antibiotic; C = surface streak method on agar containing aureomycin.
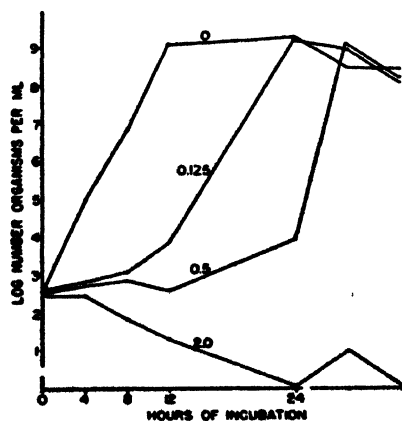
Figure 1. Effect of aureomycin on growth of *Streptococcus* 98. Each tube contained 10 ml of broth with aureomycin in the final concentrations, in µg per ml, noted on the curves and was inoculated at "0" hour with 0.1 ml of a 10⁻⁴ dilution of a 6-hour broth culture of the organism. The numbers of viable organisms were estimated at intervals by pour plates in aureomycin-free blood agar.

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this same organism in 24-hour cultures were inoculated in various concentrations of aureomycin, little evidence of antibacterial activity was manifested, even in the presence of 100 μg of the antibiotic per ml, as shown in figure 2. It would thus seem that the opportunity for vigorous multiplication must be present in order for aureomycin to exert its antibacterial activity, though the influence of the concentration of bacteria may have been a determining factor.

End points in sensitivity tests. In reading the bacterial sensitivity to aureomycin in the tube dilution test, the usual experience has been that the visual end point of growth in the tubes is lower than the end point observed when subcultures were made from the tubes to antibiotic-free agar and incubated for an additional 24 hours. Table 4 shows this difference in end points. It may be that some organisms, possibly those in the resting state, survive the 18 hours of incubation in the broth containing lethal concentrations of antibiotic, and their
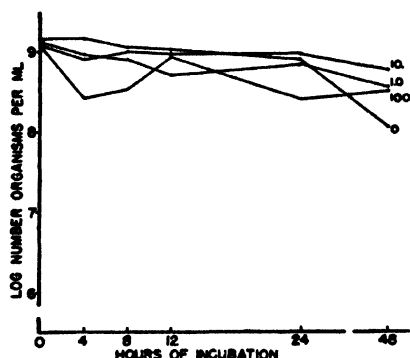
Figure 2. Effect of aureomycin on fully grown culture of *Streptococcus* 98. Each tube contained 9 ml of a 24-hour broth culture of the streptococcus, and at "0" hour was added 1 ml of broth containing sufficient aureomycin to give the indicated final concentrations in μg per ml. The numbers of viable organisms were then estimated at intervals by pour plates in aureomycin-free blood agar.

presence becomes apparent on subculture to antibiotic-free agar where growth can occur. This phenomenon may also be related to the rapid loss of antibacterial activity of aureomycin on incubation in broth.

Effect of pH. Alteration of the pH from the acid to the alkaline range resulted in a considerable decrease in the antibacterial activity of aureomycin. This effect is shown in table 5 in the case of *Bacillus* no. 5; similar results were obtained with other organisms. The decrease in activity of aureomycin in the basic pH range is in sharp contrast to the decrease in activity of streptomycin in the acid pH range (Abraham and Duthie, 1946).

Effect of added substances. The presence of a number of substances in the concentrations listed in table 6 had little or no effect on the antibacterial activity of aureomycin when they were incorporated into the broth making up the media for a tube dilution assay of the sensitivity of a test organism.

The type of solid medium employed for the surface streak sensitivity method may influence the inhibition end points obtained with aureomycin. The end

TABLE 5

Effect of pH on the antibacterial action of aureomycin

pH	READING*	CONCENTRATION OF AUREOMYCIN, μG/ML								
		0.78	0.39	0.19	0.095	0.048	0.024	0.012	0.006	0
6.1	A	0	0	0	0	0	0	0	+	+++
	B	0	0	0	0	0	0	0	+++	+++
6.6	A	0	0	0	0	0	0	+	+++	+++
	B	0	0	0	0	0	2	+++	+++	+++
7.0	A	0	0	0	0	0	0	+++	+++	+++
	B	0	0	0	0	0	+++	+++	+++	+++
7.5	A	0	0	0	+	+++	+++	+++	+++	+++
	B	0	0	0	+++	+++	+++	+++	+++	+++
8.0	A	0	0	+++	+++	+++	+++	+++	+++	+++
	B	0	+++	+++	+++	+++	+++	+++	+++	+++

Organism: *Bacillus* no. 5. Inoculum: 20,000 per ml.

0, +, ++, and +++ represent extent of growth (numbers represent number of discrete colonies).

* A = visible growth in tubes after incubation for 18 hours; B = growth on subculture of 18-hour broth culture to aureomycin-free blood agar.

TABLE 6

Effect of various substances on antibacterial action of aureomycin

SUBSTANCE	AMOUNT ADDED*	M.I.C.	AMOUNT ADDED	M.I.C.	AMOUNT ADDED	M.I.C.	AMOUNT ADDED	M.I.C.
Sodium chloride.	0	0.5	M/10	0.5	1M	—‡		
Glucose .	0	0.5	M/10	0.5	1M	0.5		
Sodium thioglycolate.	0	1.0	M/1,000	0.25	M/100	1.0	M/10	2.0
Cysteine hydrochloride	0	1.0	M/1,000	1.0	M/100	1.0	M/10	2.0‡
Semicarbazide	0	1.0	M/1,000	1.0	M/100	1.0	M/10	—‡
Urea. . .	0	0.5	M/2,000	0.5	M/200	0.5	M/20	0.5
Glutamic acid.	0	0.5	M/2,000	0.5	M/200	0.5	M/20	0.5
p-Aminobenzoic acid	0	0.5	M/800	0.5	M/200	0.5	M/80	0.25
Pteroylglutamic acid	0	0.5	M/2,000	0.5	M/200	0.5	M/40	0.5
Human plasma, pooled	0	0.6	12.5%	0.6	25%	0.3	50%	0.3

Tube dilution method; brain heart infusion broth pH 7.2 ± (Difco).

Organism: *Streptococcus* no. 98. Inoculum: ±100,000 per ml.

* Final concentration in addition to content of media.

† Minimum concentration of aureomycin for complete inhibition.

‡ Poor growth or no growth of test organism with and without antibiotic.

points observed on heart infusion agar (Difco) and on the same medium containing 10 per cent horse blood were the same; whereas the end points of aureomycin inhibition observed in the case of the same organisms on eosin methylene

blue agar were lower.   This difference may possibly be related to poorer growth on the eosin methylene blue agar.

Effect of aerobiosis.   There was no indication that sensitivity determinations made on surface streak agar plates in a decreased oxygen tension, with approximately 3.5 per cent carbon dioxide as obtained in a candle jar, altered the inhibition end points observed with the same organisms under strictly aerobic incubation.   Preliminary observations, however, suggest that greater anaerobiosis may interfere with the antibiotic action of aureomycin.

"Aureomycinase."   No evidence could be obtained that any aureomycin-inhibiting substance (similar to penicillinase) was present in filtrates of broth cultures of *Streptococcus* 98 and *Proteus vulgaris* filtered through sintered glass. These same organisms did not yield any apparent aureomycin-inhibiting material when the bacterial cells were disrupted by repeated freezing and thawing.
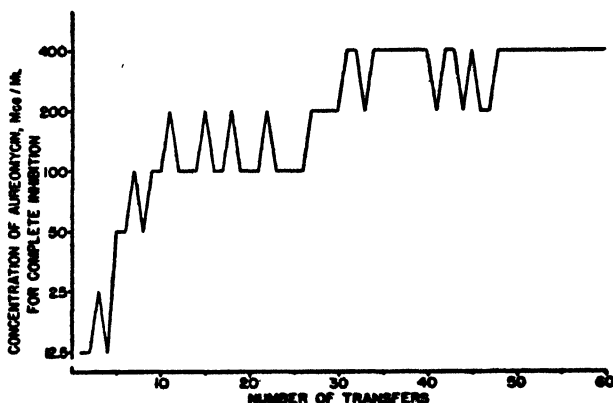
Figure 3. Development of resistance to aureomycin *in vitro.*   *Aerobacter aerogenes,* strain J.

Development of resistance in vitro.   Most of the bacteria studied did not readily become resistant to aureomycin *in vitro.*   Several strains of bacteria recently isolated from patients were subcultured at frequent intervals from the surface of 10 per cent horse blood agar containing graded concentrations of the antibiotic, to similar series of plates, using large inocula from the plates containing the highest concentration of aureomycin on which each strain had previously grown. The plates were freshly prepared and were used promptly each time.   Only one strain of *Aerobacter aerogenes,* among several organisms that were studied, showed a significant increase in resistance to aureomycin, as shown in figure 3. The same strain of *A. aerogenes,* subcultured in the same manner but in the absence of the antibiotic for a total of 60 times, showed no increase in resistance to aureomycin.   A strain of *Klebsiella pneumoniae,* type A, showed an increase in resistance to aureomycin, from 6.2 to 25 μg per ml for complete inhibition, after 42 subcultures in the presence of the antibiotic.   The latter strain subcultured for 60 times in the absence of the antibiotic showed no increased resistance to aureomycin.   Two strains of *Staphylococcus aureus,* 1 strain of *Strepto-*

coccus mitis, and 1 strain of *Streptococcus pyogenes* showed no increase in resisttance to aureomycin after 60 subcultures in the presence of the antibiotic.

Another strain of *Streptococcus mitis* apparently increased in resistance from 1.5 to 12.5 µg per ml after 21 subcultures in the presence of the antibiotic; however, the same strain transferred in the absence of aureomycin for a total of 30 times showed a similar increase in resistance, suggesting that in this case the slight augmentation in resistance was probably the result of some cultural alteration from continued subculture on the laboratory medium and not necessarily related to the exposure to the antibiotic.

In an attempt to demonstrate the possible presence of a few very resistant organisms in large populations of sensitive strains of bacteria, broth cultures of *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* were centrifuged and the sediments from 10 ml were inoculated on the surfaces of agar containing 200 to 1,000 µg of aureomycin per ml. No aureomycin-resistant or aureomycin-dependent organisms could be obtained in this manner.

The difficulty of making microorganisms resistant to aureomycin *in vitro* suggests that the development of resistance to this antibiotic *in vivo* should not be an important problem. The clinical observations made to date seem to bear this out.

ACKNOWLEDGMENTS

The aureomycin hydrochloride was supplied by the Lederle Laboratories as a dry crystalline powder in sterile sealed ampoules containing 20 mg each and in 50-mg capsules. These studies were conducted with the technical assistance of Clare Wilcox, Janice M. Bryan, and Paul F. Frank. Most of the strains of organisms from the clinical cases were isolated and identified by Marion E. Lamb and A. Kathleen Daly in the Bacteriological Laboratory of the Mallory Institute of Pathology. The strains of gonococcus were isolated by Helen Trousdale.

SUMMARY

Aureomycin has been shown to be active *in vitro* against many bacteria, including certain penicillin-resistant, streptomycin-resistant, and streptomycin-dependent microorganisms. The organisms studied have shown no marked tendency to become resistant *in vitro* on repeated exposure to aureomycin, though a moderate increase in resistance occurred in some strains. The concentration of the antibiotic required for complete inhibition of bacteria is influenced by the number of organisms present, their phase of growth, and the pH of the test media.

Aureomycin is a stable preparation as a dry powder in sealed ampoules and also in high concentrations in solution in distilled water at 4 C and 37 C. It loses potency rapidly if stored in low concentrations in broth, plasma, or in blood agar at 37 C, though it is somewhat more stable in these media at 4 C.

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PYRUVIC ACID METABOLISM

A Factor Required for Oxidation by Streptococcus faecalis

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The metabolism of pyruvic acid by lactic acid bacteria occurs through several pathways whose mechanisms and cofactors are for the most part unknown. The growth factor requirements of these organisms suggest the possibility of determining the cofactor requirements by adjusting the nutritional levels for maximum function (Bellamy and Gunsalus, 1944).

Among the enterococci, *Streptococcus faecalis*, strain 10C1, has been shown to use several organic acids as energy sources. The metabolism of citric and malic acids is adaptive (Campbell, Bellamy, and Gunsalus, 1943), whereas the metabolism of pyruvate is constitutive, although the amount of enzyme is altered somewhat by the growth conditions (Campbell, Bellamy, and Gunsalus, 1943; Gunsalus and Campbell, 1944). The growth conditions that favor the greatest rate of pyruvate oxidation and dismutation have been described by Miller (1942) and Miller *et al.* (1947).

Lipmann (1939, 1944) studied a stable pyruvic acid oxidation system of *Lactobacillus delbrueckii* (Davis strain) in some detail and found at least 5 components to be required: the enzyme protein, flavine adenine dinucleotide, inorganic phosphate, cocarboxylase, and magnesium. Manganese or cobalt could replace the latter. For pyruvate dismutation a sixth component, riboflavin, was required as a link to the lactic dehydrogenase. Inorganic phosphate was taken up during the reaction and appeared as acetyl phosphate, from which the high energy phosphate could be transferred to the adenylic acid system.

Dorfman *et al.* (1942) and McElroy and Dorfman (1948) observed that cell suspensions of *Proteus morganii* oxidize pyruvate slowly and that the rate could be stimulated by the addition of pantothenate. Later Stumpf (1945) isolated and purified a pyruvic acid oxidase from *Proteus vulgaris*, which was shown to require the enzyme protein, cocarboxylase, and magnesium. Neither an inorganic phosphate nor a pantothenate requirement was demonstrated. Pilgrim *et al.* (1942) had also shown that liver homogenates of pantothenate- or biotin-deficient rats were deficient in ability to oxidize pyruvate.

Lipmann and his associates (Novelli and Lipmann, 1947) have shown that pantothenate functions in acetylation. Thus the site of action in pyruvate metabolism appears to be beyond the oxidation reaction, specifically in the disposition of the acetate. On the basis of preliminary experiments with *Proteus morganii* McElroy and Dorfman (1948) have suggested that pantothenate is involved in the removal of acetylmethylcarbinol.

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In the present study, a synthetic medium has been obtained for the growth of *Streptococcus faecalis*, strain 10C1, with a very active pyruvate oxidase system. With the synthetic medium devised (O'Kane and Gunsalus, 1947) the enzyme occurs in the apoenzyme form and may be activated by a substance (or substances) present in yeast extract. Dried cell preparations as well as cell suspensions are activated, thus constituting another case of apoenzyme formation during cell growth (Bellamy and Gunsalus, 1945).

METHODS

Culture. *Streptococcus faecalis*, strain 10C1, from the departmental collection was stored in stock agar deeps at 5 C and transferred every 6 months. Inoculum was prepared by transferring the culture into a medium composed of 1 per cent each of tryptone and yeast extract, 0.5 per cent K_2HPO_4, and 0.1 per cent glucose. After 4 hours' incubation at 37 C, transfer was made to a second tube, which after 8- to 10-hour incubation was diluted with 20 parts of sterile distilled water and used at the rate of 0.05 ml per 10 ml of medium. If the cultures were transferred at 12-hour intervals for more than 72 hours, the growth rate in the synthetic medium decreased; therefore the culture was removed from stock every other day.

Cell suspensions. Cell suspensions were prepared from 12-hour cultures as suggested by Miller (1942)—8- to 14- hour cells gave satisfactory rates of pyruvate oxidation. The cells were harvested by centrifugation, washed once with $\frac{1}{4}$ the growth volume of M/30 phosphate buffer, pH 6.5, and resuspended in $\frac{1}{10}$ the growth volume of the same buffer.

Solutions of vitamins, accessory substances, and acid-hydrolyzed vitamin-free casein were prepared and stored as suggested by Williams and coworkers (1941). Enzyme-hydrolyzed casein (strepogenin), shown by Wright and Skeggs (1944) to stimulate the growth of several strains of streptococci including 10C1, was prepared as suggested by Roberts and Snell (1946).

The rate of oxidation expressed as Q_{O_2} (N)—microliters of oxygen taken up per hour per mg bacterial nitrogen—is based on the linear portion of the curve for the oxygen uptake, taken at 10-minute intervals for 40 minutes after the addition of substrates. The quantity of cells was estimated turbidimetrically from a previously calibrated curve.

Attempts to replace the yeast extract tryptone medium of Miller (1942) with a synthetic medium, such as that of Bellamy and Gunsalus (1945), resulted in poorer growth and cells nearly devoid of pyruvate oxidase. It was shortly found, however, that the addition of yeast extract to the Warburg cups would stimulate pyruvate oxidation by the cells from the synthetic medium. In order to obtain rapid pyruvate oxidation upon stimulation, several adjustments in the composition of the medium were necessary.

The most suitable medium found for the production of pyruvate oxidase apoenzyme is shown in table 1. The more pertinent alterations necessary to produce good growth from the synthetic medium for tyrosine apoenzyme production were higher purine and pyrimidine levels and the addition of strepogenin. The tyrosine decarboxylase medium was devised for *Streptococcus faecalis*, strain

TABLE 1

Medium for the production of pyruvate oxidase apoenzyme containing cells

INGREDIENT	QUANTITY PER 100 ML
Acid-hydrolyzed casein	1.0 g
K₂HPO₄	0.5 g
Glucose	0.3 g
Strepogenin	1,500.0 mg
Tryptophan	10.0 mg
Cystine	20.0 mg
Sodium thioglycolate	10.0 mg
Adenine sulfate	2.5 mg
Guanine hydrochloride	2.5 mg
Uracil	2.5 mg
Salts B	0.5 ml
Nicotinic acid	500.0 μg
Riboflavin	100.0 μg
Calcium pantothenate	100.0 μg
Pyridoxine hydrochloride	100.0 μg
Thiamine hydrochloride	100.0 μg
Folic acid	1.0 μg
Biotin	0.1 μg
pH	7.0–7.3
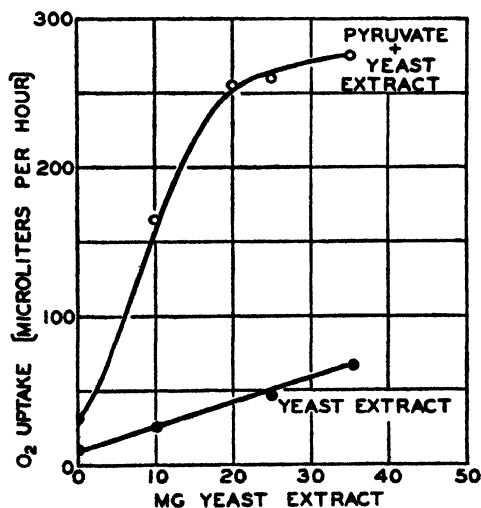
Figure 1. Activation of pyruvate oxidase apoenzyme by yeast extract. Per Warburg cup: 0.5 ml cell suspension (∞ 0.2 mg bacterial N); 2.0 ml M/20 phosphate buffer pH 6.5. Side arm: 0.2 ml M/10 pyruvate; water or factors to 0.5 ml.

R, which did not require strepogenin. In order to obtain the maximum apo-pyruvate oxidase content, levels of riboflavin, nicotinic acid, and pyridoxine were increased above those required for maximum growth, as were levels of sodium

acetate and of strepogenin. The relationship of these factors to the enzyme production is not clear beyond the previous suggestions of Lipmann (1939) that flavin is involved in pyruvate oxidation. The effect of the nicotinic acid level may be general, since it was also observed with tyrosine apoenzyme production (Bellamy and Gunsalus, 1944). The effect of pyridoxine may well be on amino acid formation (Umbreit, Wood, and Gunsalus, 1946; Lichstein, Gunsalus, and Umbreit, 1945) and protein synthesis (Stoerk and Eisen, 1946).

The stimulation of pyruvate oxidase apoenzyme by yeast extract is shown in figure 1. The rate of pyruvate oxidation by cells from the synthetic medium, here shown as 30 μl per hour, varies from one batch to another, apparently depending upon the completeness of removal of the pyruvate oxidase factor from the strepogenin preparation. Most samples of the medium yield cells in-

TABLE 2

Stimulation of pyruvate dismutation by yeast extract factor

Conditions: 0.2 mg bacterial nitrogen per cup. 2.0 ml M/30 phosphate buffer, pH 6.0. 0.2 ml M/10 pyruvate + additions as indicated tipped at 0 time. Atmosphere—N_2.

	μL CO_2 PER HOUR
Endogenous, cells alone......	8
Pyruvate, 20 μM...	8
Pyruvate + yeast extract, 20 mg...	128
Yeast extract, 20 mg	12
Pyruvate + fraction C_{12} (potency 28) 0.5 mg.................	65
C_{12} (potency 28) 0.5 mg	5
Pyruvate + fraction 19-11 (potency 200) 75 μg..	55
Fraction 19-11 (potency 200) 75 μg............. ..... .:....	5

capable of oxidizing pyruvate. The cells used in the experiment indicated showed 10-fold stimulation in activity upon the addition of yeast extract, with a maximum of about 300 μl per hour by the cells from 5 ml of medium. With yeast extract as the starting material, partial purification of the stimulant has been accomplished. These fractions were used in several experiments because they are free of an oxidizable material present in yeast extract (figure 1).

The pyruvate oxidase can, as shown by Lipmann (1939) and others, be linked via lactic dehydrogenase with a second molecule of pyruvate to yield lactic and acetic acids and CO_2. If the stimulatory effect of the yeast extract were more or less directly on the pyruvate oxidase, the cells containing the apoenzyme should also show a decreased rate of dismutation that could be stimulated by yeast extract. As shown in table 2, the deficient cells, which were unable to dismutate pyruvate, were activated by yeast extract or by the partially purified fractions. Cells from the synthetic medium are not devoid of oxidative power since they oxidize glucose rapidly and are not further stimulated by yeast extract. How-

ever, the pyruvate oxidation rate approaches that of glucose only in the presence of yeast extract (table 3).

In attempts to analyze the mechanism of the stimulatory action, deficient cells were incubated with yeast extract and both the solution and the cells assayed for the factor.   It was found that some of the factor was stored by the cells since they exhibited an increased rate of pyruvate oxidation (table 4).   Incubation of cells with pyruvate and yeast extract, under conditions that allowed pyruvate oxidation, resulted in the removal of nearly all of the stimulant from the supernatant and in a still greater rate of pyruvate oxidation by the cells.   Not all the

TABLE 3

The specificity of the yeast extract stimulation for pyruvate oxidation

SUBSTRATE	Q_{O_2} (N)
Pyruvate (20 μM)	30
Glucose (20 μM)	580
Yeast extract (20 mg).	70
Pyruvate + yeast extract	480

TABLE 4

Absorption of the factor by cell suspensions

	Q_{O_2} (N)	Q_{O_2} (N) ON PYRUVATE[*] AFTER 30-MINUTE OXIDATION	
		Cells	Supernatant
Cells + pyruvate	65	28	65
Cells + yeast extract	25	125	230
Cells + yeast extract + pyruvate	340	165	80

* Experiment as in table 3; then the cells were collected by centrifugation and resuspended in buffer and pyruvate; supernatant was returned to Warburg cup and new cells were added.

activity removed from solution, however, is found in the cells; this may be due to the destruction of the factor by hydrogen peroxide formed from pyruvate oxidation.

The accumulation of the factor in the cells may indicate a coenzyme function, the greater absorption during pyruvate oxidation suggesting a possible energy requirement for the use of the factor.   The removal of the activator from solution by the cells serves to differentiate this substance from the amino acid stimulation of pyruvate oxidation by pneumococci as demonstrated by Bernheim and Bernheim (1943).

Several compounds of known physiological activity were tested either by incorporation in the growth medium or by addition to cell suspensions (table 5). Those added to the growth medium without effect included choline, inositol,

thymine, xanthine, fumarate, citrate, and oleic acid.   No evidence of stimulation was found by either procedure with the compounds tested.   Since neither amino acids, yeast ash, nor known factors replace the yeast extract, it is suggested that an unknown factor is involved.

TABLE 5

Known substances tested for pyruvate oxidase stimulation with resting cells

	*mg/cup**
DL-Leucine.......	0.6
DL-Phenylalanine.....	0.6
DL-Methionine.	0.6
Cysteine......	1.0
Glutathione..........	1.0
Acid-hydrolyzed casein.	30.0
Wright's strepogenin..	15.0
Yeast ash........ .   . . .≈100 mg yeast extract
Folic acid....	0.03
SLR factor..... ... , ...	0.003
Pyridoxal.......	0.01
Mixture of vitamins†	
Mixtures of these vitamins with each omitted in turn	
Coenzyme A preparation‡..	0.50
Coenzyme A preparation hydrolyzed§	0.50

* Incubated at 37 C with cells 20 minutes before addition of pyruvate.

† Contained 6 μg each of pyridoxine, thiamine, riboflavin, nicotinic acid, pantothenic acid, folic acid, and 60 mμg biotin.

‡ Kindly supplied by Dr. N. O. Kaplan, 25 to 50 per cent pure.

§ Fifteen minutes at 120 C in N/2 H_2SO_4.

DISCUSSION

The observation of more specific nutritive requirements for pyruvate oxidation than for growth, and the stimulation of the pyruvate oxidation rate of cell suspensions, appears to constitute another case of the formation of an apoenzyme during growth.   Similar cases are the formation of tyrosine apodecarboxylase (Bellamy and Gunsalus, 1945) and apotransaminase (Lichstein, Gunsalus, and Umbreit, 1945).

The activation of pyruvate oxidation by resting cells, and dried cell preparations, with yeast extract though not with the known substances tested indicates that an unidentified factor may be involved.   The coenzyme nature of the activating agent is suggested by its removal from solution and storage in the cells during pyruvate oxidation.   It is, however, possible that the factor is an active form of one of the known factors that the cells are unable to synthesize.   The mechanism of action must await the identification of the active agent(s) and a study of the enzyme system involved.

Since several pyruvate oxidation enzymes have been reported (Lipmann, 1939; Still, 1941; Hills, 1943; Stumpf, 1945), speculation as to the mechanism of the action would be premature.

SUMMARY

With strain 10C1 of *Streptococcus faecalis*, the nutritive requirements for pyruvate oxidation and dismutation were found to be more specific than those for growth. Cells harvested from synthetic media that support good growth contain pyruvate oxidase in the apoenzyme form, as indicated by the activation of oxidation by cell suspensions and dried cell preparations.

Yeast extract contains a substance (or substances), apparently not identical with known accessory factors, that is required for pyruvate oxidation. This is demonstrated by the inability of cell suspensions to oxidize pyruvate unless they are grown in the presence of yeast extract, or unless yeast extract is added to the cell suspensions. The active principle is taken up by the cells during pyruvate oxidation and is retained, thus indicating the possibility of a coenzyme nature.

The oxidative deficiency is not general since the deficient cells oxidize glucose rapidly.

A synthetic medium has been obtained that supports good growth and highly active pyruvate oxidase apoenzyme production.

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NOTES

A NOTE ON THE SUSCEPTIBILITY OF HEMOPHILUS INFLUENZAE TYPE B TO BACITRACIN

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Bacitracin is a new member of the group of antibiotics produced by the gram-positive sporeforming bacilli. It was first reported by Johnson, Anker, and Meleney in 1945 (Science, **102,** 376), and it is produced by a member of the *Bacillus subtilis* group of organisms. Investigation of this antibiotic is being conducted under a grant from the United States Army. The bacitracin is being supplied by the Commercial Solvents Corporation.

Many organisms isolated from pathological processes have been tested for sensitivity to bacitracin. It has been observed that most of the gram-negative bacilli were resistant to the action of the antibiotic. An exception was noted in the case of four strains of *Hemophilus influenzae* which were obtained from the spinal or ventricular fluid in four cases of meningitis in humans. These strains were all type B, and were freshly isolated from the pathological material. Three of the strains were tested for susceptibility to bacitracin on primary culture of spinal or ventricular fluid as well as on subcultures. These constituted the only strains of this organism available for study; without exception they appeared to be sensitive to the antibiotic.

The pathological specimen suspected of containing *Hemophilus influenzae* was planted on 10 per cent human blood agar in petri plates. A heavy inoculum was spread evenly over the plate with a bent wire. A piece of filter paper approximately 1 cm square was soaked in a solution of bacitracin containing 20 units per ml and placed in the center of the plate. After 24 to 48 hours' incubation, the squares were surrounded by an inner zone of complete inhibition outside of which was an indefinite area of sparsely scattered colonies. This merged with the dense growth on the remainder of the plate. Subcultures were tested in a similar manner. Approximately the same results were obtained by using steel penicylinders filled with the antibiotic. One of the strains was tested for sensitivity in a tube assay. The organisms were grown and titrated in blood extract broth. This strain was inhibited by 0.625 to 0.63 units of bacitracin per ml under these conditions.

A SATISFACTORY MEDIUM FOR THE ISOLATION, CULTIVATION, AND MAINTENANCE OF VIABILITY OF VIBRIO FETUS (BOVINE)

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The strain of *Vibrio fetus* that this laboratory has found in aborted bovine fetuses during the past two years is more difficult to grow in laboratory media and to keep viable without transfer at close intervals than strains that were isolated 15 to 20 years ago.

In an attempt to find a more suitable culture medium for *Vibrio fetus*, a new medium known as "thiol," developed by the Difco laboratories, was studied. This is semisolid medium containing 0.1 per cent agar. It was developed for the purpose of growing aerobic and anaerobic organisms in the presence of high concentrations of penicillin or streptomycin.

Thiol medium, if prepared according to the directions of the manufacturer and if the pH is adjusted to 6.8 before sterilization, will grow vibrios rapidly and in large numbers when present in the exudate of the stomach of an aborted fetus. The vibrios grow profusely at or within 0.5 mm of the surface of a tube of the medium. Maximum growth is obtained in a tube (8-by-1-inch) of medium in 4 days at 37 C from inoculation of a pure culture of vibrios. If one desires to obtain a dense suspension of vibrios for antigenic studies, the organisms may be harvested from the top layer of the tube of medium at intervals of 4 days. On microscopic examination the appearance of the vibrios varies from short S curves to long spiral filaments.

It has been found that the vibrios will remain alive in tubes of thiol medium held at room temperature (26 C) for at least 150 days without intervening transfers.

OBSERVATIONS ON THE GROWTH OF PSITTACOSIS VIRUS IN CHORIOALLANTOIC MEMBRANES BY ELECTRON MICROSCOPE

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It is the purpose of this paper to present observations obtained with the aid of the electron microscope on the development of psittacosis virus in the embryonated egg. The first phase of the problem was to study the adsorption of virus on the cell surface and its subsequent penetration into the cell. The development of the infectious process was then pursued by studying infected tissue disrupted by sonic vibration and infected allantoic fluids harvested after the growth cycle was completed.

METHODS AND MATERIALS

The virus studied was the 6BC strain of psittacosis virus, originally isolated by Dr. K. F. Meyer and adapted by repeated passage to grow well in the allantoic cavity of embryonated eggs. Harvests of allantoic fluid were made after 5 to 6 days' incubation and the virus was purified by centrifugation.

Titrations for infectivity of virus samples were performed by the single-dilution method (Golub, 1948), in which the LD_{50} end point is calculated from the average day of death of a group of eggs inoculated with one dilution by the yolk-sac route.

Chorioallantoic membranes of 9-day-old eggs were infected by inoculation on the surface through a hole in the side of the egg where a false air sac had been formed. The inoculum consisted of 0.5 ml of a 10 X concentrated, purified suspension of allantoic fluid virus or, in the case of controls, of 0.5 ml of sterile buffer solution. The shell holes were sealed with a cellulose-acetate mixture and the eggs incubated at 37 C. At the intervals of harvest the shell was broken away to the edges of the false air sac and the exposed portion of the chorioallantoic membrane removed with the aid of forceps and scissors.

Antisera were prepared in rabbits either by 1 inoculation of partially purified, living yolk-sac virus or 5 inoculations of active mouse liver and spleen virus by the intraperitoneal route. The rabbits were bled 2 weeks after the last inoculation and the serum was stored in a dry ice chest.

The surfaces of normal and infected chorioallantoic membranes, dried on the formvar-coated glass slides, were studied by the silica replica method. A description of the technique for replica preparation and of the method for rotary shadow-casting employed will be published elsewhere.

The chorioallantoic membranes were placed in a solution of 0.10 M NaCl and 0.02 M phosphate buffer at pH 7.0 and subjected to sonic vibration. After 15 minutes most of the suspension was removed and salt-buffer solution was added. The remaining fragments of the membrane were then vibrated another 15

the allantoic fluid of eggs inoculated into the allantoic cavity also suggested a rapid adsorption of the virus onto the surrounding tissue.   Eggs were inoculated

TIME OF CONTACT BETWEEN VIRUS & MEMBRANE
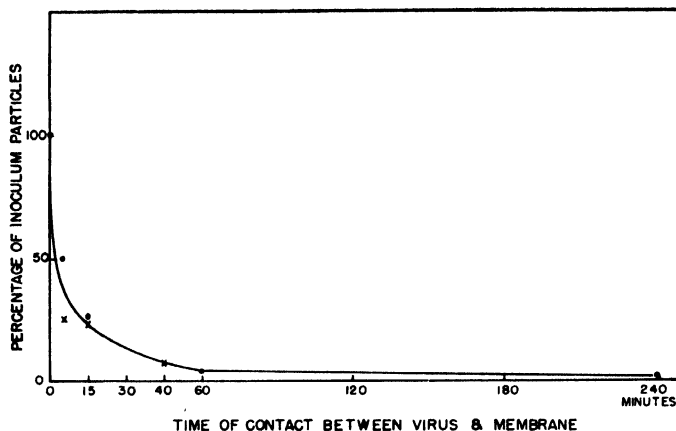
Figure 2A Percentage of decrease in particle count of membrane surface inoculum at various intervals.

TIME OF INCUBATION
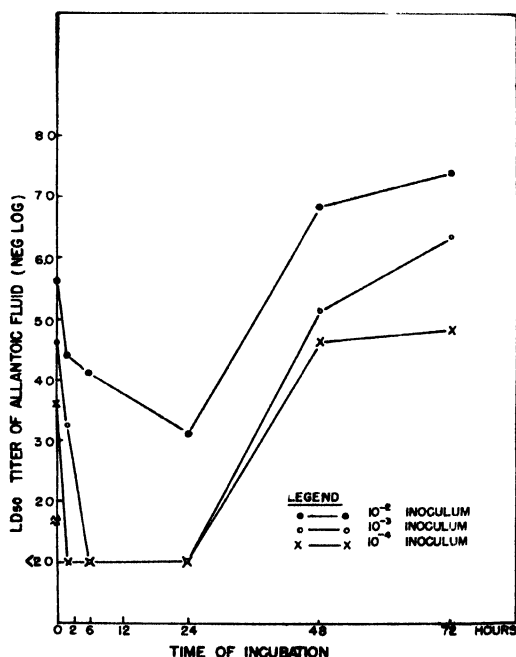
Figure 2B. Infectivity titer of allantoic fluid harvests at various intervals after inoculation with psittacosis virus.

with 10^{-2}, 10^{-3}, and 10^{-4} dilutions of a stock 6BC psittacosis virus and at intervals of 2, 6, 24, 48, and 72 hours pooled samples of fluids from five living eggs of

each group were tested for virus activity. The titer at zero time for each inoculum was calculated from the known titer of the virus and the estimated dilution factor involved. The results are shown in figure 2B.

It can be seen that the infectivity titer of the fluids dropped rapidly within a few hours of inoculation. Control experiments have shown that simple incubation of virus *in vitro* for short intervals does not result in comparable decreases in titer. The less rapid drop with increase in inoculum concentration would suggest that the saturation point of cells susceptible to virus attachment was being reached.

Studies on sonic-wave-vibrated normal chorioallantoic membranes. Electron microscopic studies on vibrated normal membranes revealed many structural components of the cell and gave the impression of the very complex nature of the cellular organization. Comparative studies on normal and infected membranes were made, and only those structural elements that seemed to be associated with the development of the psittacosis virus were extensively investigated. The significance of the comparative study of normal and infected membranes became apparent after repeated observations disclosed some modes of virus development appearing to be integrated with normal growth of the intracellular elements. For this reason, a few examples of structural elements of the normal membranes following sonic vibration are presented.

Electron microscopic observations on vibrated normal membrane suspensions reveal a large number of dense particles having a broad size distribution. A striking feature is the large number of fibers interconnecting various bodies and often originating in large masses from such bodies. In figure 3A is shown an example of dense intracellular particles, suggestive of nuclear material. Figure 3B shows a more irregular particle with a less dense border area, and in figure 3C are examples of the frequent association of particles with bundles of fibers. Individual fibers exhibit transverse striations and some variation in their dimensions. A few fibers were measured and were found to be in the range of 34 mμ to 56 mμ; the distance between the striations was, on the average, 64 mμ.

Fibers were also observed in suspensions of ground membranes and therefore cannot be considered as a phenomenon resulting from vibration. Furthermore, replica studies on membranes, where a layer of tissue was pulled off by an attached plastic film, revealed that such fibers are normally present in the cell.

Studies on sonic-wave-vibrated infected chorioallantoic membranes. Electron microscopic studies were performed on vibrated membranes at 6, 12, 24, 48, and 72 hours after infection, a period considered to cover the complete range of virus development. At the same time, studies were also performed on corresponding control membranes that had been inoculated with sterile buffer solution in place of the virus suspension.

Six-hour infected membranes did not show any visual difference in structural elements from the controls. The 12-hour infected samples showed some isolated particles that closely resembled the typical virus particle. Twenty-four, 48-, and 72-hour membranes showed increasing numbers of viruslike particles and aggregates not seen in the controls.

Both infected and normal samples contained a large number of forms of vary-
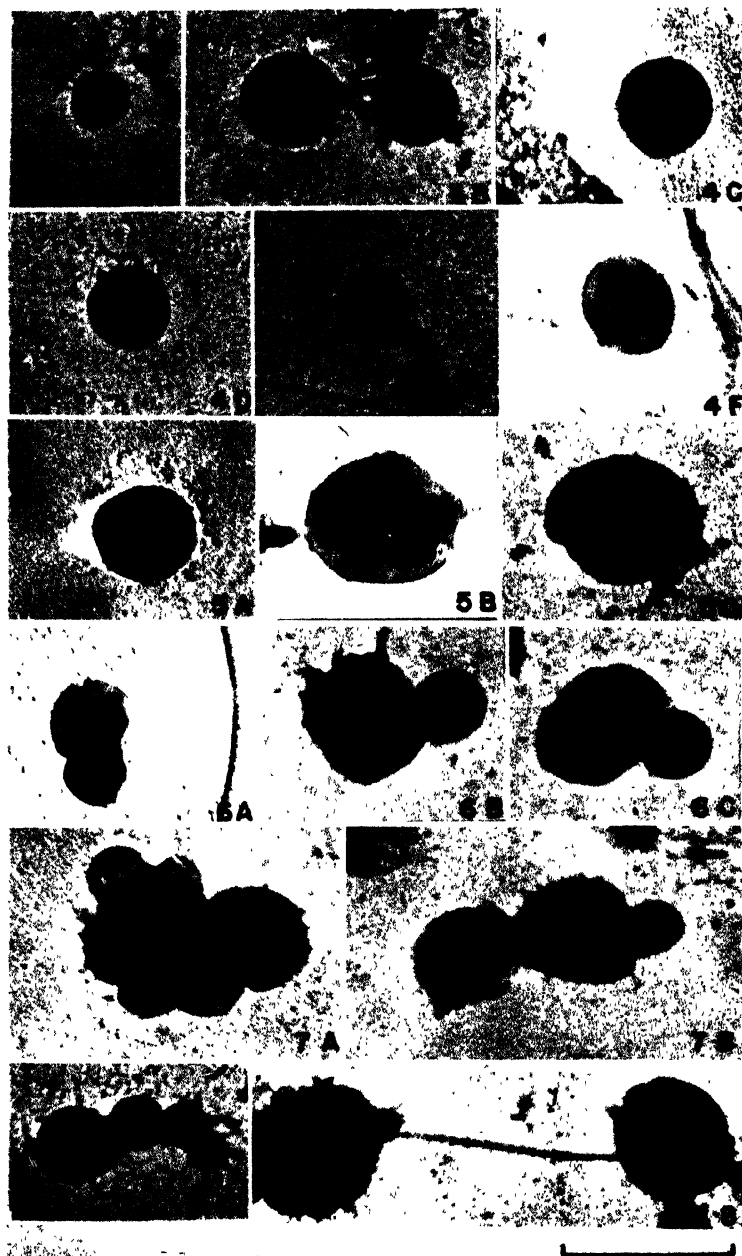
Figures 4A-8.

Figures 4-13. Structures observed in sonic-wave-vibrated, infected chorioallantoic nembranes.
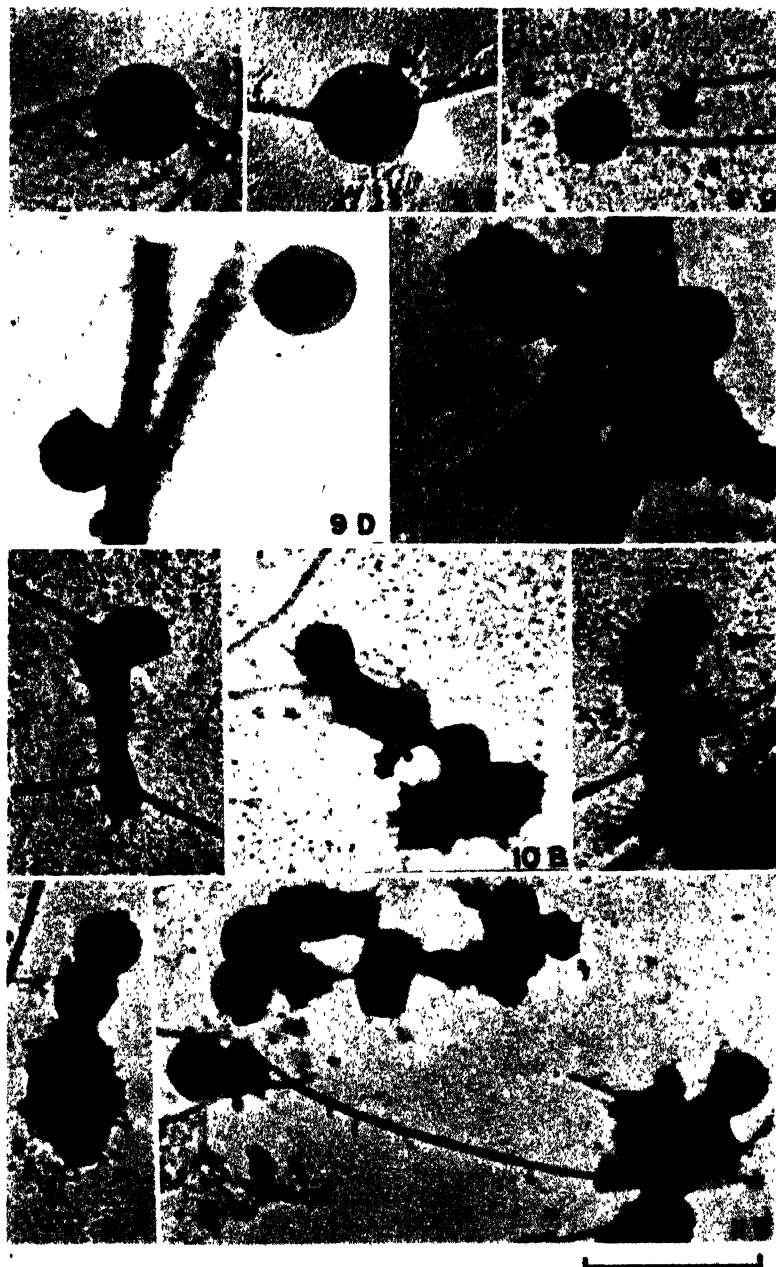
Figures 9A–11 B.

Figures 11C-13
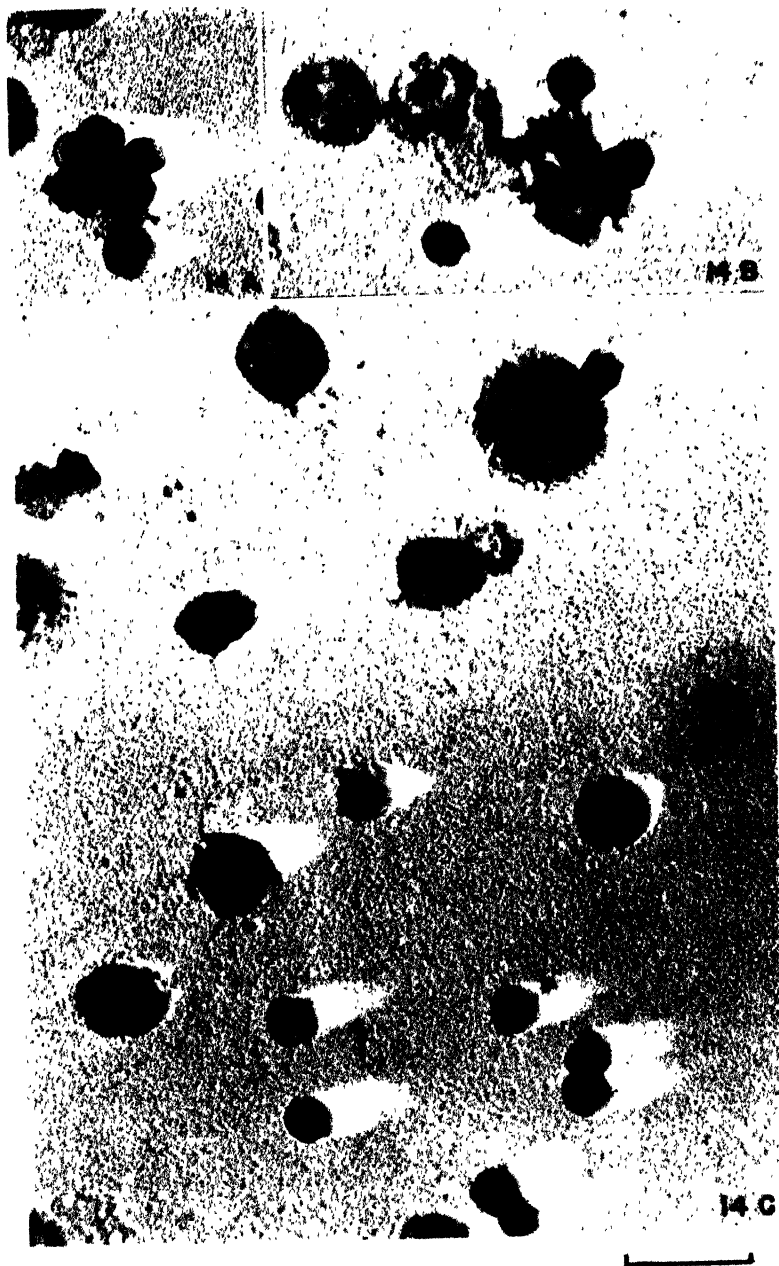
Figure 14. Purified preparation of infected allantoic fluid, 5-day-old culture.
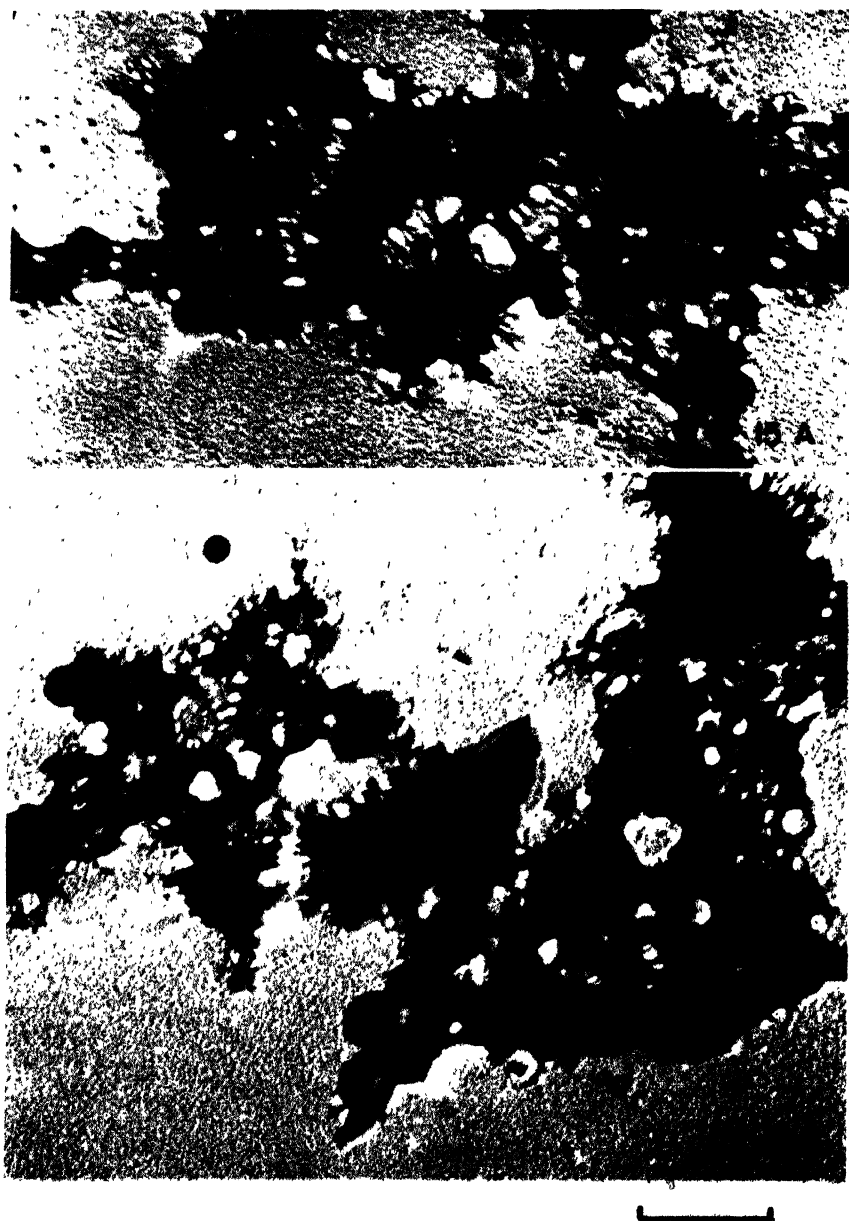
Figure 15. Psittacosis virus bodies agglutinated in the presence of specific immune serum.

Note: Stationary shadow-casting was performed with gold at an angle tangent 1/5. Scale length under each group of electron micrographs is 1 micron. One-micron scale lengths for different magnifications are given directly on the print.

individual particles located in the border region of the aggregate are clearly visible, as demonstrated in figure 13. Repeated observations indicate that such aggregates are heavily loaded with virus particles.

Studies on virus bodies from allantoic fluid preparations. In figure 14 are shown the normal bodies in the varying morphology, as seen in many similar preparations of psittacosis partially purified from infected allantoic fluid. It was at first felt that the variations from the small, typical body were elementary bodies undergoing degradation. However, examinations of incubated samples for periods up to 10 days indicated that the proportion of the large, flat forms did not increase, although definitely visible morphological degradation of both forms
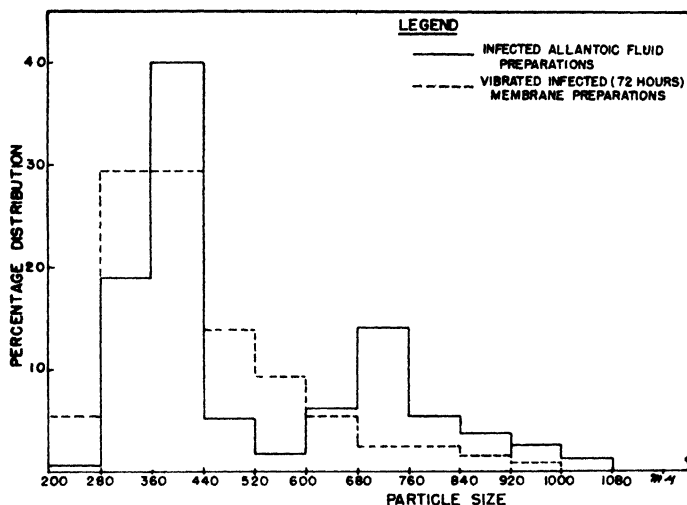
Figure 16. Size distribution of particles from infected allantoic fluid and chorioallantoic membranes

was apparent. It is believed, therefore, that the large flat bodies are not direct degradation forms of the small elementary body.

To test the relationship of these large forms to the smaller elementary bodies, the purified virus suspension was examined after incubation for 3 hours with its specific immune serum. Figure 15 shows clumps of the bodies visible in this preparation, and it is seen that the large, flat cells were agglutinated along with the small, centrally dense bodies, indicating that the former are probably the actual virus in a morphologically different form. Strands of serum protein material are plainly evident and in some areas coat the virus aggregates so heavily as to mask completely the typical morphology of the individual particles.

Size distribution of virus particles. It was of interest to compare the size distributions of virus bodies originating from allantoic fluid with those obtained from the vibrated infected membranes. About 150 particles of each series were

measured and distribution curves calculated. The total count included both the small particles and the large flat bodies. Distribution data in graphical form are presented in figure 16.

The virus sample prepared from 5- to 6-day incubated allantoic fluid cultures shows two distinct size regions. The first maximum peak is in the range between 300 mμ to 440 mμ and represents particles with a dense central region and a diffuse border area. The second maximum is in the range of 650 mμ to 800 mμ and is made up largely of diffuse flat bodies. On the other hand, virus originating from 72-hour infected membranes shows only one distinct maximum peak, but there are more particles in the size range between the two peaks of the allantoic fluid material.

DISCUSSION AND SUMMARY

The initial phase of psittacosis virus infection of the host cell is in all probability an adsorption of the infectious particle on the cell surface. With regard to subsequent events, we have always assumed, actually without much direct evidence, that penetration of the cell membrane follows, in which internal environment the virus particle finds the proper conditions for its multiplication process.

Our observations on the disappearance of virus bodies from an inoculum on the surface of the chorioallantoic membrane, as measured by particle counts in the electron microscope, revealed that after 4 hours' contact with the tissue, only about 1 per cent of the original virus bodies remained suspended in the fluid, the rest presumably having formed an attachment to the membrane. The studies on the rapid decrease in activity titer of allantoic fluid inoculated with psittacosis virus *in vivo* confirm the rapid adsorption of the infective particles on the surface of susceptible tissue. Membrane replica studies of infected tissue, in which virus particle imprints practically disappeared 5 hours after inoculation of the membrane, suggest that the succession of events has progressed beyond the adsorption phase and that actual penetration is almost complete by this time.

It was revealed that the shape of the imprints of viruslike particles was actually changing from elevations to cavities and holes when observations were made at various intervals following inoculation, and this is construed as suggesting actual cell penetration. No visible differences were observed between normal membrane replicas and the 12-hour infected samples, further suggesting that the sites of virus invasion on the membrane surface had undergone repair by this time.

The fate of the virus particles after penetration of the cell membrane has been the subject of various speculations. The numerous light microscopic observations are not in complete agreement as to the multiplication process in all details, but one view is that the virus is capable of immediate multiplication without the formation of large bodies. This possibility is considered to be dependent upon intracellular environmental influences (Burnet and Rountree, 1935; Levinthal, 1935; Yanamura and Meyer, 1941). The more common and generally accepted alternative mode of psittacosis virus multiplication is the formation of large bodies from smaller particles and dispersion of the latter by a process of

disintegration resulting in the liberation of various-shaped smaller particles. The formation of a dense matrix around the seat of virus multiplication was considered to be an intracellular reaction of the host cell to virus invasion and constituted an accumulation of protoplasmic material (Bedson, 1933, 1934; Bland and Canti, 1935; Burnet and Rountree, 1935; Levinthal, 1935; Yanamura and Meyer, 1941). Observations on the multiplication of some other viruses reveal, in general, similar developmental modes. The observations of Tang and Wei (1937) on the multiplication of vaccinia virus suggest that this virus may possess two independent methods of multiplication. The first is by simple division, giving rise to new particles with varied morphology. The second process of multiplication is considered to be a combined action of the invading virus and the host cell, under which conditions large particles with accompanying matrix formation are observed. Somewhat similar observations were made by Rake and Jones (1942) on the development of lymphogranuloma venereum virus.

Our first observations with the electron microscope were performed on psittacosis virus purified from allantoic fluid. The two distinct morphological forms, one smaller, centrally dense particle, and another larger, flat body were revealed (figure 14). The size distribution curve had two distinct maxima (figure 16), and the ratio of the two particle types varied in the samples. Agglutination experiments indicated that both particles had antigenic factors in common. Our studies on viruslike particles originating from infected membranes reveal the same over-all size range as in virus from allantoic fluid, but the distribution of particle sizes is different. Morphologically, there is great similarity between particles seen in allantoic fluid (figure 14) and those obtained from vibrated infected membranes (figures 4 and 5). These findings suggest that we are probably dealing, in both cases, with the same type of virus particles, the principal difference being that in a 72-hour infected membrane the morphological development of the virus is, on the average, in an earlier phase than are those originating from 5- to 6-day cultures of allantoic fluid.

In our experiments virus particles from vibrated membranes were not apparent until 12 hours after infection. In spite of extensive observations only a few isolated particles were found that resembled the typical virus particle contained in the inoculum. The fact that the 6-hour infected membranes did not reveal any such virus particles indicated that the particles visible in subsequent samples were new growth and not original inoculum. Light microscopic and electron microscopic observations are apparently in agreement as to the time necessary for the first cycle of virus multiplication.

Our observations on later samples also revealed large, dense particulate aggregates, varying in size and density, on whose border areas were exposed many virus forms (figure 13) and which may correspond to a colony of virus. Many of the aggregates were probably distorted by the sonic vibration, but in general they manifested considerable resistance, indicating that the particles within the aggregates are firmly embedded.

It appeared to us that the virus in certain instances associates itself with various normal intracellular elements and possibly follows a pattern of development

similar to the normal reproduction of these components. Speculating along lines upon which others (Zinsser, 1937; Stanley, 1939; Stanley, 1941; Soule, 1940) have theorized, it may be that certain forms of the normal intracellular structure, once they have been in direct contact with an invading virus body, develop the potentiality themselves to produce virus at the seat of contact or induce virus. growth in associated elements. This would then be merely an altered reproduction of the normal cellular constituents resulting from the new pattern induced by the virus. Virus growth as just described is simply an abnormally rapid multiplication process of modified normal intracellular structural elements within a certain limited size range. It may be akin to malignant tissue growth, wherein abnormal reproduction of apparently normal cells takes place. The stimulus for the latter is, in most instances, unknown, but in the case of the viruses would be the presence of living virus particles carrying the growth-inducing property. It will be recalled that even in virus infections one of the earliest manifestations is stimulation of cell growth in the vicinity of the affected area.

The question still remains as to whether direct virus multiplication can be initiated without association with some structural elements of the cell. Light microscopic observations, due to insufficient resolving power, can only indicate that multiplication proceeds without large particle formation. Electron microscopic observations, although of increased resolving power, have the disadvantage that they are performed on dried specimens and the objects of study are, of necessity, removed from their intracellular environment. Virus body combinations were seen in both vibrated membranes (figures 6, 7, and 11) and allantoic fluid preparations (figure 14), a fact which suggests that simple division occurs, although not necessarily equal division. Similar observations were made by Rake et al. (1946) on the development of lymphogranuloma venereum virus in the yolk sac. However, these forms may previously have been connected to large forms or to intracellular elements and become separated later. In most of these observations, the dense central material of the elementary body seems to play a significant role in the process.

Further uncertainty exists concerning the function and significance in virus multiplication of the two major type virus bodies, i.e., the small body with the dense center and the larger flat body. Repeated observations create the impression that there are usually one or several small dense particles associated with a large body and suggest that the flat body gives rise to smaller dense forms, following which it disintegrates (figure 14A, B). The earlier shape of free flat bodies is also uncertain, but our impression is that some type of the centrally dense particles (figure $4D$, E, F) is a precursor of the flat forms. The phenomenon of rearrangement in the shape of the dense central material seems to be one step of virus multiplication, and further developmental forms, e.g., figure $10B$, C, result in chain formation, as in figure $7C$. Further suggestive evidence is seen in a comparison between the size distribution curves of particles originating from 5- to 6-day allantoic fluid preparations and the 72-hour infected tissue. The former has two distinct particle size maxima, but the latter shows more of the intermediate and smaller forms. This suggests that intermediate forms, after

further development and with rearrangement of the dense central portion, give rise to other centrally dense particles in the smallest size region, after which they appear as flat bodies, and thus might be considered as late stages of the so-called initial bodies. Our impression is that the "wrinkled pea" appearance (Rake et al., 1946) of the elementary body with the irregular central mass is not an artifact due to the drying process, but is caused by the uneven distribution of this material in the virus body at certain stages in its development. The form of this material appears to change with the cycle from a rounded mass, almost filling the body, to a more irregular, ameboid-shaped form. In the later stages it becomes dispersed in scattered areas throughout the large body and finally disintegrates almost entirely, resulting in the flat forms.

In brief summary then, incorporting previous views, the following modes of psittacosis virus development in the egg are suggested: the virus bodies are adsorbed rapidly onto the cell surface and soon penetrate the cell wall, leaving holes in the membrane which are quickly repaired. Some of the virus particles may have the potentiality to multiply directly, provided the environmental conditions are suitable. This does not exclude the possibility, however, that the virus must contact certain normal structural elements of the cell before it acquires the ability to reproduce itself. It is suggested that one route of development may proceed by association with or incorporation into normal cellular constituents, following which either the normal cellular elements then produce the altered material recognized as virus or the virus itself gains therefrom the capacity to reproduce in kind.

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STUDIES ON STREPTOMYCIN

III. The Effect of Streptomycin on the Metabolism of Resting Bacteria and on Certain Purified Enzymes

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There has been very little experimental work on the mechanism by which streptomycin (SM) exerts its activity on living cells or the reason for the resistance of some bacteria to its action. Several reports have compared the metabolic activities of strains susceptible and resistant to SM (Graessle and Frost, 1946; Murray *et al.*, 1946; Seligmann and Wassermann, 1947). Some investigators (Benham, 1947; Hirsch and Dosdogru, 1947; Bernheim and Fitzgerald, 1947; Geiger, 1947) have failed to obtain SM inhibition of the oxidation of carbohydrates or carbohydrate intermediates by bacteria susceptible to SM. On the other hand, with certain bacteria SM has been shown to inhibit the oxidation of benzoic acid (Bernheim and Fitzgerald, 1947), amino acid metabolism (Geiger, 1947), and the oxidation of sodium ribonucleate (Krampitz, Green, and Werkman, 1947).

The work reported in this paper is concerned with the effect of SM on (a) certain purified enzyme systems, (b) aerobic and anaerobic oxidations by resting cell suspensions of *Staphylococcus aureus*, *Bacillus cereus*, and *Shigella sonnei*, and (c) the production and utilization of certain intermediate and end products resulting from aerobic metabolism.

METHODS AND MATERIALS

For the experiments with purified enzymes, carboxylase was prepared from dried brewer's yeast (Green, Herbert, and Subrahmanyan, 1941), carbonic anhydrase was prepared from sheep blood (Tauber, 1936), and a rat heart preparation (Stotz and Hastings, 1937) was used for the study of the succinoxidase and cytochrome-cytochrome-oxidase systems. Manometric techniques were employed for measuring the activities of the following enzymes: rabbit blood catalase, carboxylase (Green, Herbert, and Subrahmanyan, 1941), urease (Sizer, 1939), carbonic anhydrase, succinoxidase, and cytochrome-cytochrome-oxidase with hydroquinone as substrate. The method used for measuring tryptic activity was that of Anson (1938). The cytochrome-cytochrome-oxidase system in intact baker's yeast was studied by the spot plate test described by Sevag and Ross (1944). There was ½-hour contact between the enzymes and SM before measurement of

activity, with the exceptions of urease and trypsin with which the activity was measured immediately after the SM was added.

Strains of *S. aureus*, *B. cereus*, and *S. sonnei*, sensitive to approximately 1 μg SM per ml, were used in the bacterial studies. Variants resistant to 1,000 μg per ml were obtained by serial transfer in broth containing increasing concentrations of SM and were studied concomitantly with the parent-susceptible strains. The *S. aureus* suspensions used were prepared from 18-hour growth in a modified Landy and Dickens (1942) semisynthetic medium at 37 C. The *B. cereus* and *S. sonnei* cells were prepared from 18-hour growth in nutrient broth. All flasks were inoculated from 24-hour agar slants of the cultures. Constant volume Warburg respirometers were used in the manometric experiments. The manometers were shaken through a to-and-fro distance of 3.5 cm at a rate of 110 cycles per minute. All experiments were run in duplicate and were repeated at least once.

For the aerobic experiments the cell suspensions were centrifuged, washed twice with M/60 Sorensen's buffer, pH 7.2, and resuspended in the same buffer to the appropriate turbidity. In the study of endogenous respiration 12 mg (dry weight, *in vacuo* over H_2SO_4) of bacteria were used per flask, and the O_2 consumption was measured over a period of 24 hours at 36.5 C. The respiratory rate of the controls slowly decreased over this time period to about 10 per cent of the initial rate. When substrates were added the cell suspensions were diluted so as to consume approximately 100 to 200 mm^3 O_2 per hour. The Warburg flasks contained a final concentration of M/60 Sorensen's buffer, pH 7.2 in most cases, 0.5 per cent substrate, and 1 to 3 mg cells. In all cases substrates were added as the neutral salts. The inner cup contained 0.2 ml 20 per cent KOH to absorb CO_2. In all manometric experiments the SM was placed in the side arm and added to the system after an equilibration period of approximately 15 minutes in the water bath at 36.5 C. Oxygen consumption was measured over a period of 3 hours.

In one series of aerobic experiments, chemical determinations were made of certain intermediate and end products of metabolism found in the cell environment and derived from various carbohydrate substrates, both in the absence of SM and in the presence of inhibitory concentrations of SM. Substrate concentrations and amounts of cells were chosen in these experiments so that approximately two-thirds of the initial substrate had been utilized by the end of the 3-hour observation period. At the end of the experiments, the cells and supernatants were separated by centrifugation and the chemical tests performed.

Colorimetric methods were used for the determination of glucose (Somogyi, 1945), pyruvate (Bueding and Wortis, 1940), lactate (Barker and Summerson, 1941), and ethanol (Henry et al., 1948). Since even purified SM interfered with the glucose test, the supernatants were always diluted for this determination to reduce the SM concentrations below the level giving interference. Glycerol was determined by a technique developed in this laboratory in which the formaldehyde produced upon splitting the glycerol with periodic acid is determined colorimetrically after reaction with 1,8-dihydroxynaphthalene-3,6-disulfonic acid

(chromotropic acid). Acetate was determined by the steam distillation and titration procedure of Elliott *et al.* (1942). The titration values were converted to acetic acid, although any volatile acid will be determined by this method. The reducing substances in the cells were determined as glucose by the method of Somogyi (1945) following hydrolysis in 3 N HCl at 100 C for 3 hours.

In the anaerobic Warburg experiments the cell suspensions were washed twice with 0.117 per cent $NaHCO_3$ (the concentration necessary to give a pH of 7.2 when equilibrated with the CO_2-N_2 mixture used, 93.5 per cent N_2 + 6.5 per cent CO_2) and resuspended in the same $NaHCO_3$ solution to the appropriate turbidity. The Warburg flasks contained a final concentration of 0.117 per cent $NaHCO_3$, 0.5 per cent substrate, and from 1 to 3 μg cells. CO_2 production was measured over a period of 3 hours.

The cells for the Thunberg experiments were prepared by washing them twice with M/30 phosphate buffer, pH 7.2, and resuspending them in the same buffer. The tubes contained a final concentration of 0.5 per cent substrate (M/50 in the case of amino acids), 0.002 per cent methylene blue, and 100 μg SM per ml in the M/30 phosphate buffer. The cells were placed in the cap. The tubes were evacuated to 20 mm Hg with an ordinary water aspirator for 3 minutes while in a water bath at 40 C. The cells were then mixed with the contents of the tube and reduction time was measured.

The SM preparations used in this work included the following: streptomycin sulfate, Pfizer commercial, lot no. 456; streptomycin hydrochloride, Merck commercial; streptomycin sulfate, Pfizer lot no. S7117A, 830 μg streptomycin base per mg; streptomycin sulfate, Parke, Davis, 800 μg streptomycin base per mg. The dihydrostreptomycin, lot no. 411 × 156, assay 585 μg per mg, was supplied to us by Parke, Davis and Company.

Many of the substrates were kindly supplied by E. S. G. Barron, Department of Medicine, University of Chicago, and by J. F. Owings, Jr., W. N. Nutter, and H. E. Thompson of Camp Detrick, Maryland.

<div align="center">RESULTS</div>

Purified enzyme systems. The activities of rabbit blood catalase, yeast carboxylase, urease, carbonic anhydrase, trypsin, succinoxidase, and the cytochrome-cytochrome-oxidase systems were unaffected by 500 μg SM per ml. The catalase activity of *S. aureus* and the cytochrome-cytochrome-oxidase system of intact baker's yeast were uninhibited by 500 and 1,000 μg SM per ml, respectively.

Endogenous respiration. SM in concentrations up to 2,000 μg per ml had no effect on the rate of endogenous O_2 consumption of *S. aureus* during the 24 hour observation period. The addition of ribonucleic acid (Schwarz Labs., Inc.) to the reaction system had no effect either in the presence or absence of SM.

The endogenous O_2 consumption of the susceptible strain of *B. cereus*, however, was inhibited by even 1 μg SM per ml. The endogenous rate of the resistant strain of *B. cereus* was inhibited only slightly by 100 μg SM per ml. The endogenous R.Q. of the susceptible strain of *B. cereus* was found to be 1.0 in

the absence of SM, indicative of carbohydrate oxidation. In the presence of 10 μg SM per ml, the R.Q. was 1.4, indicating that SM alters the endogenous metabolism.

Aerobic oxidation. In the case of the sensitive strain of *S. aureus*, inhibition of glycerol and lactate oxidations was observed with as little as 1 μg SM per ml, whereas no inhibition occurred with the resistant strain with 100 μg SM per ml (table 1). SM in the concentration of 100 μg SM per ml did not inhibit the oxidation by the sensitive strain of the following substrates: glucose, fructose, ethanol, pyruvate, ascorbate, sodium salt of adenylic acid, glutamate, D-ribose, α-glycerophosphate, glyceraldehyde, dihydroxyacetone, glycerate, and glyoxal. Among the substrates not utilized were succinate, fumarate, butyrate, citrate, acetaldehyde, stearate, glycolate, and glyoxylate. Acetate was utilized oc-

TABLE 1

The effect of streptomycin on the aerobic oxidation of glycerol and lactate by S. aureus

SUBSTRATE (0.5%)	STRAINS	SM	AVERAGE % INHIBITIONS DURING TIME PERIODS (HR) INDICATED			
			0–0.5	0.5–1	1–2	2–3
		$\mu g/ml$				
Glycerol	S	1	0	10	14	20
		10	0	15	26	32
		100	0	23	35	42
	R	100	0	0	0	0
		1,000	0	0	0	20
Lactate	S	10	0	7	15	20
		100	0	14	22	25
	R	1,000	0	0	0	0

S = susceptible strain; R = resistant strain.

casionally at a very slow rate; when utilized the oxidation was inhibited completely by 100 μg SM per ml.

With the sensitive strain of *B. cereus*, 1 μg SM per ml produced inhibition of oxidation with all the substrates studied, namely, glycerol, lactate, glucose, pyruvate, ethanol, and acetate. The inhibitions were usually quantitatively greater than those obtained with *S. aureus;* in fact the oxidation of acetate was completely blocked by 1 μg SM per ml. The concentrations of sodium acetate used were 0.054 and 0.027 per cent, both of which gave identical results. Utilization of this substrate began at a slow rate during the second hour and approached a maximum during the third and fourth hours (QO_2 = 28). No utilization occurred even at 4 hours when a concentration of 0.5 per cent was used. Appreciably less or no inhibition of the oxidation of these substrates by the resistant strain was produced with 100 μg SM per ml.

In experiments with the sensitive strain of *S. sonnei* inhibition by 10 μg SM

per ml was obtained with glycerol, lactate, pyruvate, succinate, and acetate, but not with ethanol. No inhibition occurred with the resistant strain at a concentration of 100 μg SM per ml.

A comparison between the susceptible and resistant strains of the QO_2 values for each substrate revealed that in some cases there was no significant difference and in the remainder of cases there were as many increased values for the resistant strains as there were decreased values. Furthermore, there was no consistency in these changes between the different organisms.

In almost every instance in which inhibition appeared it did so only after a lapse of time. The inhibition usually increased with time, and the lag period before the appearance of inhibition generally decreased with increasing concentration of SM (cf. table 1). One possible cause for this lag is that time is required for SM to reach the site of action (perhaps a question of cell membrane permeability) or to adsorb onto that site to a sufficient degree to cause the inhibition. Experiments were conducted to test this postulate. SM was incubated for 1 hour with cells of S. aureus prior to the addition of substrate (glycerol, lactate). The resultant inhibition appeared at the same time and to the same degree as in experiments in which the SM was added to the cells at the same time as the substrate.

The inhibitions observed apparently are not of the competitive type. Within experimental error the degree of inhibition obtained with several substrates with B. cereus and S. aureus was the same over a 10-fold range of substrate concentrations.

It was realized that these inhibitions might be a reflection of the bactericidal action of SM. If such is the case, the inhibition-time curve should follow the same course as the viable cell count, time curve. The viable cell count of B. cereus, however, remained fairly constant after an initial drop during the first 2 hours' contact with 10 μg SM per ml. As indicated by turbidity measurements, there was no lysis during the course of the experiments. Furthermore, with S. aureus and S. sonnei, the oxidation of several substrates was not inhibited.

It has been pointed out (Brooks, 1947) that one possible cause for the inhibition of O_2 consumption by inhibitors is an altered permeability of the cell membrane to O_2 or the exogenous substrate. Since inhibitions were observed under anaerobic conditions, it seems unlikely that the inhibitions under aerobic conditions are a reflection of altered permeability to O_2. Altered permeability to exogenous substrate was apparently ruled out by experiments in which it was shown that SM did not affect the permeability of susceptible cells to pyruvate (unpublished data).

It was reasoned that if SM blocks a respiratory chain at a specific point, then the substrate for that particular enzyme should accumulate. Tables 2 and 3 are balance sheets showing the effect of SM on the aerobic carbohydrate metabolism of B. cereus and S. aureus, respectively.

In the calculation of these tables it was assumed that the endogenous respiration continues uninfluenced by the presence of added substrate, and the O_2 con-

sumption values in the presence of substrate were corrected accordingly. In experiments with acetate as substrate for *B. cereus* SM completely blocked the utilization of acetate (determined by chemical analysis at the beginning and end of the experiments) and reduced the rate of O_2 consumption to that of endogenous controls containing the same SM concentration. Especially with *B. cereus* and

TABLE 2

Balance sheet showing the effect of streptomycin on the aerobic carbohydrate metabolism of B. cereus

SUBSTRATE	SM	% OF THEORETICAL O_2 CONSUMPTION	OVER-ALL % INHIBITION OF O_2 CONSUMPTION	OVER-ALL % INHIBITION OF SUBSTRATE UTILIZATION	R.Q. Theor.	R.Q. Observed	Glucose	Pyruvate	Lactate	Glycerol	Ethanol	Acetate	CO_2	GLUCOSE INCREASE IN CELLS EXPRESSED AS % C*	TOTAL % RECOVERY
	μg/ml														
Glucose	0	37			1.0	1.0		0.46	1.2		0	6.9	37	3.9	50
	1	39		13		1.0		0.23	6.9		0	11	38	4.3	60
	10	43	21	31		1.0		2.1	5.4		0	28	43	1.8	80
Pyruvate	0	50			1.2	1.3	0				0	0	16	52 → 1.1	69
	1	53	27	30		1.3	0				0	0	26	56 → −0.6	81
	10	44	41	34			0				0	0	45	47† → −1.8	90
Lactate	0	55			1.0	1.1	0	0			0	5.8	54	2.5	63
	1	52	10	13		1.1	0	0			0	14	49	0.12	63
	10	47	30	14		1.1	0.62	0			0	21	44	−2.0	64
Glycerol	0	39			0.86	0.72		‡	4.1		0.50	26	33	2.2	66
	1	42	14	16				‡	6.3		0.60	49	34	1.3	91
	10	36	36	32		0.70		‡	8.5		0.85	48	31	0	88
Acetate	0	50			1.0	0.95							43	3.9	47
	1		100	100											
	10		100	100		0.95								−3.2	

Each value is the average of 2 to 6 experiments run in duplicate.

* Change during experiment in total cell-reducing substances not related to endogenous metabolism, determined as glucose, and expressed as percentage of C relative to the total C in the substrate utilized.

† Where an R.Q. value is not given, the R.Q. obtained with the other concentration of SM is assumed.

‡ A violet-red color developed in these determinations unlike the color given by pyruvate. The substance present causing this is unknown.

its relatively high endogenous rate, if this correction is not made certain values in the balance sheets would be changed quantitatively but not qualitatively. Since interpretations are made only from the qualitative nature of these results the validity of this assumption is not critical.

In instances in which an interfering substance was found to be present for a particular analysis and it was not possible to make a correction for this inter-

ference, the analyses were omitted from the data given in tables 2 and 3. Tests for some of the substances in the supernatants from endogenous systems were positive although the amounts found were small. These values were subtracted from those obtained for the supernatants containing initial substrate.

Along with the determinations of reducing substance in the cells at the end of the experiment, an aliquot of cells stored at 4 C during the experiment was analyzed. The total reducing substance in the cells of the endogenous controls at the end of the experiment was usually considerably less than that in the ali-

TABLE 3

Balance sheet showing the effect of streptomycin on the aerobic carbohydrate metabolism of S. aureus

SUBSTRATE	SM	% OF THEORETICAL O₂ CONSUMPTION	OVER-ALL % INHIBITION OF O₂ CONS.	OVER-ALL % INHIBITION OF SUBSTRATE UTILIZATION	R.Q.		RECOVERY AT END OF EXPERIMENT, EXPRESSED AS % C RECOVERED FROM SUBSTRATE USED						GLUCOSE INCREASE IN CELLS, EXPRESSED AS % C*	TOTAL % RECOVERY
					Theor.	Observed	Pyruvate	Lactate	Glycerol	Ethanol	Acetate	CO₂		
	µg/ml													
Glyc-	0	39			0.86	0.73	1.0	1.2		1.6	51	38	1.9	95
erol	10	36	16	8			1.2	3.2		1.8	57	35†	2.0	100
	100	42	31	36		0 73	1.3	1.5		1.8	63	38	2.7	108
Lactate	0	32			1.0	0.75	5.2		0		35	22	0	62
	10	34	12	16				10	0		49	24	−2.3	81
	100	29	24	19		0.72		16	0		52	19	0	82
Glucose	0	26			1.0	1.0	3 0	5.2			30	27	0.50	66
	100	26	0	0		1.0	3.0	4.5			30	27	0.50	65

Each value is the average of 2 to 6 experiments run in duplicate.

* Change during experiment in total cell-reducing substance not related to endogenous metabolism, determined as glucose, and expressed as percentage of C relative to the total C in the substrate utilized.

† Where an R.Q. value is not given, the R.Q. obtained with the higher concentration of SM is assumed.

quot stored at 4 C. This undoubtedly represents utilization of stored carbohydrate reserves. This was confirmed by the observation of an R.Q. of 1.0 for endogenous O₂ consumption with *B. cereus*. Since in all other calculations it has been assumed that endogenous metabolism is unaffected by the presence of exogenous substrate, the value for the total reducing substance found in the endogenous cells at the end of the experiment was taken as the reference point from which the reducing substance in the cells exposed to exogenous substrate either increased or decreased.

The columns in tables 2 and 3 headed by "% of theoretical O₂ consumption" represent (observed O₂ consumption per theoretical O₂ consumption for complete oxidation of substrate utilized) × 100. This "theoretical O₂ consumption" is seldom realized, and in some reports it has been adequately demonstrated

that a considerable part of the fraction of substrate not oxidized to completion is synthesized to a carbohydrate and retained by the cell. It has been found (Clifton, 1946) that in the presence of appropriate concentrations of certain inhibitors, e.g., azide, the O_2 consumption approaches the theoretical for complete oxidation and the synthesis of the carbohydrate is blocked. As shown in the tables, the percentage of theoretical O_2 consumption did not increase in the presence of SM. These experiments were not allowed to go to completion, and, as seen in the tables, certain substances tested for appeared in increased amounts in the presence of SM. It was considered possible that if these substances (and probably others not tested for) were permitted sufficient time to be metabolized, the percentage of theoretical O_2 consumption might have increased. Several experiments were run with *B. cereus*, using glucose, glycerol, and lactate as substrates, to test this possibility. The experiments were continued at least 1 hour after the substrate was exhausted. The percentage of theoretical was not increased either in the presence of inhibitory or subinhibitory (0.1 μg per ml) concentrations of SM.

The columns headed "Theoretical R.Q." represent R.Q. values for complete oxidation of the substrates to CO_2 and H_2O. The columns headed "Observed R.Q." represent average values for 3-hour experiments, and are not corrected for endogenous respiration. If the corrections are made, the values are changed by a maximum of 5 per cent. Two facts appear upon studying these columns: first, SM in inhibitory concentrations does not affect the R.Q.'s; second, with but two exceptions (glycerol as substrate for both organisms, and lactate as substrate for *S. aureus*), the observed R.Q.'s do not differ significantly from the theoretical values. Since in no instance was a substrate oxidized completely to CO_2 and H_2O, in order for the R.Q. not to change, the total C, H, and O in the other substance or substances formed from the substrate must be in the same or very nearly the same ratio as in the substrate. Failure of the observed R.Q. to conform with the theoretical can only mean the contrary. The oxidation of glycerol by *S. aureus* offered an opportunity to determine whether the observed R.Q. was compatible with the over-all reaction indicated by the chemical analyses for intermediates and end products in a case in which the observed and theoretical R.Q.'s differed, and in which approximately all of the C in the substrate utilized was accounted for. The over-all reaction as indicated by the balance sheet (table 3) gives an R.Q. of 0.72, which agrees very well with the observed R.Q. of 0.73. This close agreement also indicates that at least in this instance the volatile acid determined was acetic.

All the quantities of the various substances tested for in the supernatants at the end of the experiments as well as the reducing substance (determined as glucose) in the cells were converted to C. These C values were then referred to the C which disappeared as the initial substrate. These percentages of C recoveries are listed for the various substrates with and without SM in tables 2 and 3. These values varied somewhat from experiment to experiment but the trends were constant, and since no interpretation is to be made from their exact magnitude, the averages of several experiments are given in the tables.

Acetate was the only substance tested for that was found to be present consistently in higher amounts in the presence of SM than in the absence of SM. This held true for all substrates with both organisms. Acetate also was found to accumulate in increased amounts during the endogenous respiration of the susceptible strain of *B. cereus* in the presence of inhibitory concentrations of SM. As seen in table 2, both the oxidation and utilization of acetate by the susceptible strain of *B. cereus* were completely blocked. In the case of the resistant strain, neither the oxidation nor the utilization of acetate was inhibited by 100 μg SM per ml.

One possible explanation for the inhibition of the oxidation of substrates other than acetate, with *B. cereus*, which was considered was the following: as acetate accumulates the equilibria in the enzymatic chain are shifted until a point is reached at which the over-all rate is decreased. Experiments were run to test this possibility. Acetate in a concentration approximating that recovered at the end of other experiments was added with glucose. The inhibition of O_2 consumption developed at the same time and to the same extent whether or not acetate was present. These results would appear to rule out this possibility.

Examination of the data in the columns headed "Glucose increase in cells, expressed as % C" reveals that with one exception (*S. aureus*, lactate as substrate) the total hydrolyzable reducing substance of the cells increased in the absence of SM. These increases, however, are considerably smaller than those reported for similar experiments with other microorganisms such as yeast (Winzler, 1940). With *B. cereus*, inhibitory concentrations of SM appeared to block this increase partially or completely. With *B. cereus*, therefore, SM apparently blocked the oxidative assimilation of carbohydrate. The percentage of theoretical O_2 consumption did not increase concomitantly because of accumulation of substances such as acetate in the supernatant. With *S. aureus*, however, there was no indication of such a block.

The last columns of the tables show the total percentage of C recovered in the substances tested for (including the CO_2 produced) from the C disappearing as substrate. In only one case (glycerol as substrate for *S. aureus*) was all of the C accounted for. This was to be expected since unquestionably there are many more intermediates formed in the metabolism of these substrates than were tested for. It is quite possible that some of these also accumulate in the presence of SM.

Anaerobic metabolism. Since the multiplication of the three susceptible strains under strictly anaerobic conditions was inhibited by SM, it was of interest to determine whether SM also inhibited the metabolism of any substrates anaerobically. Of the substrates utilized by *S. aureus*, no inhibition was observed in any case. With *B. cereus*, inhibition of the anerobic metabolism of glucose and pyruvate was produced by 1 μg SM per ml. With *S. sonnei*, 1 μg SM per ml inhibited the metabolism of pyruvate. Again when inhibition occurred, usually there was a lag period before it developed. In all cases the resistant strains were unaffected by 100 μg SM per ml.

Differentiation between metabolic CO_2 and acid production (both being meas-

ured as CO_2 liberated under anaerobic conditions) was made in the following three cases (Umbreit et al., 1945): in the oxidation of glucose by B. cereus and S. sonnei the CO_2 was acid CO_2; in the oxidation of pyruvate by S. sonnei approximately 70 per cent of the CO_2 was acid CO_2 and 30 per cent metabolic CO_2 In the latter case the production of both acid and metabolic CO_2 was inhibited by SM.

Using S. aureus, the anaerobic metabolism of the following substrates was studied by the methylene blue Thunberg technique: glucose, fructose, ethanol, glycerol, succinate, pyruvate, lactate, acetate, citrate, malate, cis-aconitate, xanthine, stearate, DL-isoleucine, L-leucine, DL-alanine, β-alanine, DL-phenylalanine, DL-valine, L-lysine HCl, DL-lysine diHCl, L-arginine, L-glutamate, DL-methionine, glycine, L-tryptophan, and L-asparagine hydrate. In no case was any inhibition observed; in many instances there was stimulation. In view of the lag period observed before inhibition of respiration develops in the Warburg respirometers, inhibition might not be expected in the Thunberg tube since the reaction was complete within 10 to 20 minutes. Furthermore it was found that, when methylene blue in a concentration that even slightly inhibits oxygen uptake (using a substrate the oxidation of which can be inhibited by SM) is added to the Warburg flasks, as much as 100 μg SM per ml produce no further inhibition. This too might explain why no inhibition was obtained in the Thunberg tube since the controls and the tubes containing SM all contained methylene blue in an inhibiting concentration.

With B. cereus the following substrates were studied: glucose, glycerol, lactate, pyruvate, ethanol, acetate, and succinate. There was no inhibition.

Indications that the inhibitions observed are due to SM. Since it is possible that the inhibitions might be produced by impurities in the samples of SM, several different preparations of varying purities were used. The inhibitions were the same with commercial SM-sulfate, commercial SM-hydrochloride, and highly purified SM-sulfate, Pfizer, and SM-sulfate, Parke, Davis. SM inactivated by being boiled 30 minutes in 0.5 N NaOH did not produce inhibition. Dihydrostreptomycin produced the same inhibition of glycerol oxidation by S. aureus as SM-sulfate, Pfizer.

In vitro, the maximal activity of SM occurs at approximately pH 7.8, and decreases markedly below pH 7.0 (Wolinsky and Steenken, 1946). A comparison of the pH activity curves of SM on the multiplication of the sensitive strain of S. aureus and its aerobic oxidation of glycerol and lactate revealed superimposable patterns.

In correlation with the antagonistic effect of salts on SM inhibition of bacterial multiplication (Berkman, Henry, and Housewright, 1947), salts were found to antagonize the metabolic inhibitions. The phosphate buffer concentration in the respirometers was found to have a direct relation to the degree of inhibition observed. For example, in the oxidation of lactate by B. cereus, in M/7.5 buffer no inhibition occurred with 100 μg SM per ml, but in M/120 buffer 50 per cent inhibition occurred with as low as 1.0 μg per ml. Inhibition of the oxidation of pyruvate in M/7.5 phosphate buffer reached 40 per cent with 10 μg SM per ml,

whereas in M/120 buffer inhibition reached 70 per cent with 10 μg per ml. A M/120 phosphate buffer was adequate for maintaining the neutral pH in the respirometers. Salt added to the system after inhibition of respiration had begun did not reverse the inhibition. This might indicate that these inhibitions are irreversible.

DISCUSSION

Numerous quantitative differences were observed in QO_2 values and anaerobic CO_2 production between the susceptible and resistant strains of the three organisms studied. No indication as to the cause for resistance or the mode of action of SM, however, can be derived from these differences since none was consistent among the three organisms. The fact that these changes do not occur consistently in the same direction would indicate that the interpretation of Seligmann and Wassermann (1947) that resistant strains have "damaged" enzyme systems is unwarranted.

The inhibitions of metabolic functions produced by SM seldom reached 100 per cent (excepting acetate as substrate with *B. cereus*), indicating either that some intermediate reaction is only partially blocked or that the step that is blocked is in parallel with others not affected. In every instance studied, acetate (or a similarly volatile acid) accumulated more in the presence of inhibitory concentrations of SM than in its absence. The fact that the oxidation of acetate by the sensitive strain of *B. cereus* was very susceptible to SM might account for the increased accumulation of acetate in this case. With *S. aureus*, however, there is evidence that the greater accumulation in the presence of SM resulted from greater production of acetate in the presence of SM. First, *S. aureus* oxidized acetate at a very low rate. Second, with glucose, a substrate the oxidation of which was not inhibited, more acetate did not accumulate in the presence of SM, although acetate was one of the end products of its metabolism. This increased production of acetate could result from SM blocking an alternate pathway that does not have acetate as an end product.

SM might inhibit the functioning of an enzyme in a carbohydrate reaction chain by combining with it specifically. The lag period usually observed for the development of the inhibition would seem to favor an alternative hypothesis. The lag period may represent the time during which some substance essential for the metabolism is being depleted and not being replenished (other intrepretations were rejected in an earlier section). SM would then be blocking the formation of this essential substance (enzyme? coenzyme? substrate?). The observed inhibitions of oxidative metabolism would thus be an indirect consequence of the primary inhibition.

Whether or not the inhibitions of metabolic functions observed bear a causal relationship to the bacteriostatic action of SM cannot be said from these experiments. Several observations were made which, although not constituting direct evidence for a causal relationship, are, however, compatible with such a hypothesis. These observations are as follows: First, the inhibitions in the susceptible strains were brought about in most cases by concentrations of SM

that just cause bacteriostasis. Second, the same functions in resistant strains were affected appreciably less or not at all by much higher concentrations of SM. Third, the pH-activity curves for SM are very similar in the two cases. Fourth, salts reverse both phenomena. Fifth, SM's of varying purity produced the same degree of inhibitions, indicating that the inhibitions probably were not due to one or more impurities present. This is further evidenced by the failure of SM inactivated by heat and alkali to produce the inhibitions.

SUMMARY

Streptomycin in very high concentrations does not inhibit catalase, carbonic anhydrase, cytochrome-cytochrome-oxidase, succinoxidase, carboxylase, urease, or trypsin.

Streptomycin in concentrations just bacteriostatic noncompetitively inhibits the metabolism of certain carbohydrate intermediates in susceptible strains of *Staphylococcus aureus*, *Bacillus cereus*, and *Shigella sonnei*. Resistant strains of these organisms were little or not at all affected by high concentrations of streptomycin.

It is postulated that streptomycin either inhibits an enzyme or enzymes involved in carbohydrate metabolism or inhibits their formation. This inhibition, in both cases studied in this investigation, resulted in increased accumulation of acetate.

Whether or not the inhibition of metabolic functions observed bears a causal relationship to the bacteriostatic action of streptomycin cannot be concluded from this study, although all the observations made are at least compatible with such a theory.

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FACTORS INFLUENCING HISTOPLASMIN FORMATION

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Histoplasmin was originally defined by Zarafonetis and Lindberg (1941) as "the antigenic substance or substances of *Histoplasma capsulatum*" Darling. The term, however, is now used generally to indicate the filtrate derived from the growth of the mycelial phase of *Histoplasma* in a liquid synthetic medium and capable of producting a positive skin reaction in sensitized animals and man. The histoplasmin that was prepared by Zarafonetis and Lindberg and that produced cutaneous reactions in experimentally infected rabbits was a filtrate from a 7 weeks' growth of the mycelial phase in Williams' synthetic medium (Williams and Saunders, 1934). Van Pernis, Benson, and Holinger (1941) used a filtrate from about 1 month's growth of the mycelial phase in a glucose broth and obtained cutaneous reactions in an infected human patient and in experimentally infected mice. Emmons, Olson, and Eldridge (1945) reported the preparation of histoplasmin in the synthetic broth medium used by Smith (1943) for the preparation of coccidioidin. The particular lot of histoplasmin used most extensively by Emmons *et al.* (1945) and by Palmer (1945) was grown in a dark cupboard at room temperature for 7 months. A similar preparation was used by Christie and Peterson (1945) in their studies on histoplasmosis.

Although histoplasmin has been used extensively for intradermal testing, little is known about the process by which the antigen is produced and what factors are most influential in its formation. Accordingly, a study was initiated to determine what factors play important roles in the development of histoplasmin. This paper reports experiments on the influence of the environment and of nutritive substances in the medium on the formation of histoplasmin.

MATERIALS AND METHODS

The nine strains of *H. capsulatum* used in this study were obtained from the collection matintained in this laboratory. The stock cultures were kept on Sabouraud's glucose agar at room temperature.

The asparagine glucose medium (Smith, 1943) with which experiments were started had the following composition:

L-Asparagine	14.0	g
Glucose (cerelose)	10.0	g
Glycerin	25.0	g
Magnesium sulfate·7H$_2$O	1.5	g
Dipotassium phosphate (K$_2$HPO$_4$)	1.31	g
Sodium citrate·5½ H$_2$O	0.9	g
Ferric citrate (scales)	0.3	g
Water to make	1,000	ml

This medium was dispensed usually in 250-ml Erlenmeyer flasks in 100-ml amounts and inoculated with a small piece of mycelium from a Sabouraud agar slant. The cultures were incubated at room temperature for varying lengths of time, at the end of which periods the broth was decanted and filtered through a Mandler filter.

The antigenic potency of the sterile filtrate was determined by one or both of two different methods: (a) the complement-fixation test was conducted (Salvin, 1947b) with sera from rabbits hyperimmunized with cells of the yeastlike phase (Salvin and Hottle, 1948) and from rabbits hyperimmunized with a suspension of ground mycelium and histoplasmin; (b) the intradermal tests in rabbits and guinea pigs were conducted as described by Emmons et al. (1945).

In the early phases of the work, the antisera used in the complement-fixation tests were obtained from rabbits hyperimmunized with histoplasmin and formalized ground mycelium. Later, however, when large quantities of the yeast-like phase became available (Salvin, 1947a), rabbits were similarly hyperimmunized with formalized yeastlike cells. Antigens assayed with antisera obtained from the latter consistently showed higher complement-fixing titers, although four units of each serum were used in all cases. Relatively low titers of the filtrate from the asparagine glucose medium (1:64 to 1:512) appeared with serum from the rabbits immunized with histoplasmin and ground mycelium; the high titers (1:4,000 to 1:16,000) with serum from the rabbits immunized with the yeastlike phase. The explanation for this phenomenon is not known.

EXPERIMENTS AND RESULTS

Relationship between skin-test activity and complement-fixation antigenicity. In order to learn whether the power of histoplasmin to produce a skin reaction was related to its power to fix complement in the presence of immune serum, tests were conducted on different lots of histoplasmin. As shown in table 1, those filtrates that did not induce skin reactions in guinea pigs did not fix complement in the presence of immune serum; those that induced only weak skin reactions had low complement-fixation powers; and those that produced good skin reactions displayed high binding power in complement-fixation tests. When this parallelism between the two tests was shown to exist and since the complement-fixation test was capable of being more accurately controlled than the skin test, complement fixation was chosen as the method for evaluating the activity of the culture filtrates in this study.

Biochemical changes in asparagine glucose medium during growth of H. capsulatum. Strain 6510 of *H. capsulatum* was grown in 100-ml quantities of the asparagine glucose medium in a series of 250-ml flasks in the dark at room temperature. At intervals the series was sampled by filtering the contents of individual flasks, and the sterile filtrate was assayed with regard to (a) hydrogen ion concentration, (b) concentration of reducing sugars, (c) total nitrogen content, and (d) complement-fixing power (figure 1).

During the first two weeks no significant change was noted in any of the four factors under study. However, at the end of this period, the growth of the

mycelium was sufficient to produce measurable changes in the medium. The power of the filtrate to fix complement in the presence of immune serum increased perceptibly, reaching a peak titer of 1:512 at the end of the seventh week. This

TABLE 1

Relationship between skin-test titer and complement-fixation titer of histoplasmin

	STRAIN	SKIN REACTION*		1/COMPLEMENT-FIXA-TION TITER†
		Dilution 1:10	Dilution 1:100	
1	6510	0	—	0
2	6510	0	—	0
3	6510	13.5	0	8
4	6510	17.0	0 (<5)	32
5	6510	—	14.0	128
6	6510	—	13.3	256
7	6510	—	13.3	256
8	6507	—	2.0‡	0
9	6507	—	10	100
10	6508	0	0	0

0 = no titer.   — = not tested.

* Average diameter (in mm) of oedematous area in at least four sensitized guinea pigs.

† Antigens titered against serum from rabbits hyperimmunized with histoplasmin and ground mycelium.

‡ Three out of four pigs were negative.
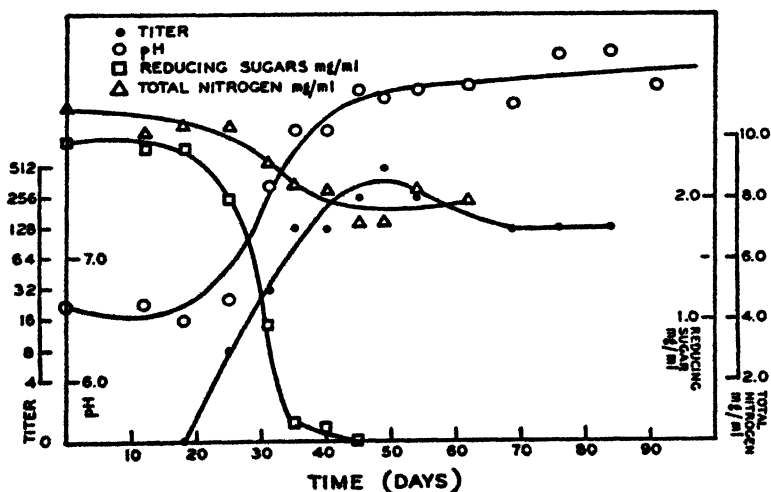
Figure 1. Changes in the culture medium following inoculation of strain 6510 of *H. capsulatum* and incubation at room temperature (25 C).

was followed by a slight decrease to a titer of 1:128 at the end of 2½ months of growth. The pH rose from about 6.6 to about 8.4 during the next 4 weeks and remained at about 8.5 during the rest of the test period. The reducing sugars

showed an abrupt drop from about 9.6 mg per ml to 0.4 mg per ml, although all the sugars were not utilized until the forty-fifth day after inoculation. The total nitrogen did not change significantly until 4 weeks after inoculation, at which time there was a gradual decrease, which continued for the next 2 weeks. The quantity of nitrogen in the medium thereafter remained the same, namely, about two-thirds of its original value.

Influence of carbohydrates on histoplasmin formation. Studies were made of the growth of strain 6510 in 100-ml quantities of the asparagine medium containing 1 per cent of one of 27 different carbohydrates in addition to the glycerol already in the basal medium (table 2). The highest titers (1:64,000 and 1:128,-000) after 9 weeks of growth were observed in the media containing cellobiose,

TABLE 2

Effect of carbohydrates on the production of histoplasmin by strain 6510 of H. capsulatum

	COMPLEMENT-FIXATION TITERS* AFTER GROWTH OF		
	4 wk	6 wk	9 wk
No sugar......................	0	0	512
Glucose.......................	0	4,000	16,000
Cellobiose	1,024	8,000	64,000
Dextrin.......................	256	16,000	64,000
Glycogen......................	32	16,000	128,000
Starch....	128	4,000	128,000

Galactose, inulin, lactose, maltose, mannitol, raffinose, sucrose, and trehalose produced titers approximately the same as those of glucose. Adonitol, dulcitol, erythritol, inositol, mannose, melezitose, melibiose, rhamnose, and sorbitol produced titers perceptibly lower than those of glucose. Arabinose, levulose, salicin, and xylose produced no titers during the first 9 weeks of growth.

* The serum used in determining these complement-fixation titers was obtained from rabbits hyperimmunized with formalin-killed cells of the yeastlike phase.

dextrin, glycogen, or starch. These four carbohydrates also were among those that supported development of the antigens as early as 4 weeks after inoculation. The values obtained after 9 weeks' growth in the media with the four aforementioned carbohydrates were considerably higher than those in the medium without any carbohydrate, and significantly higher than those in the medium with glucose. In all carbohydrate media in which good mycelial growth was evident, the antigenic titer was equal to or greater than that of the medium without sugar.

Optimal environmental conditions for histoplasmin formation. The asparagine glucose medium was inoculated with the mycelium of strain 6510 and exposed to differences in hydrogen ion concentration, light, temperature, surface-volume ratio, and depth of growth. Of these factors, optimal conditions for the production of histoplasmin involved the use of a medium at an initial pH of 7.0 to 8.0, with a surface-volume ratio as near unity as feasible, and at a temperature of incubation of about 25 C. The degree of light and the depth of growth seemed to have no measurable influence on the production of histoplasmin.

Strain variations in histoplasmin formation. When eight strains of *H. capsulatum* were compared for histoplasmin production, seven of the eight formed the antigen at about the same rate and in the same amount by the end of the growth period, i.e., about 2 months. One strain grew more slowly than the others and was correspondingly slow in its production of histoplasmin.

DISCUSSION

Although skin testing has been used in the past for determining the activity of histoplasmin, the complement-fixation technique was employed in this study. That the complement-fixation test is valid for this purpose was demonstrated by preliminary parallel tests of activity of histoplasmin used as a skin-testing and as a complement-fixation antigen. In fact, the complement-fixation test is believed to be more satisfactory for assaying antigens, because (1) the reagents are standardized for each test and can be accurately measured, (2) animal variations are eliminated, and (3) large numbers of titrations can be conveniently made.

The complement-fixation test has made possible the study of histoplasmin formation during the growth of the mycelium. Thus, the age of the broth filtrate was found to be important, since its titer varied from zero at 3 weeks to a maximum at 7 to 8 weeks. Simultaneously with the appearance of histoplasmin, several changes in the medium were noted, namely, a sharp rise in pH, a marked and rapid decrease in reducing sugars, and a gradual decrease in total nitrogen in solution. A possible interpretation of these phenomena is that as the mycelium grows nitrogen is removed from the broth to form protoplasm and the glucose is converted to a nonreducing substance, which processes are accompanied by an increase in alkalinity due to ammonia formation. By the end of the growth period (2 months after inoculation), weights of dry mycelium as high as 800 mg per 100 ml of culture medium have been found (Salvin and Hottle, 1947).

When the standard asparagine medium was altered by substituting various carbohydrates for the glucose, a remarkable difference in the titers of the broth filtrates was noted, varying from 0 to 1:128,000 after 9 weeks of growth. Of interest is the fact that the highest titers were obtained in media in which the carbohydrate was a polymer of glucose and, with the exception of cellobiose (a disaccharide), a polyhexose.

Since the production of histoplasmin varies so greatly under different conditions of growth, each preparation for either skin testing, complement fixation, or precipitin testing should be carefully assayed before use. In this way, observations with different lots of histoplasmin may be more readily compared and evaluated.

SUMMARY

A complement-fixation test was used to evaluate the effect of environmental and nutritive factors on the formation of histoplasmin by the mycelium of *Histoplasma capsulatum* Darling.

A maximum yield of histoplasmin in a glucose asparagine medium was obtained after 8 to 10 weeks of growth at room temperature. The appearance of histo-

plasmin in the medium was paralleled by a rise in alkalinity to pH 8.0 to 8.5, a drop in reducing sugars to zero, and a 25 per cent decrease in total nitrogen.

Optimal conditions found for histoplasmin formation involved a medium with an initial pH of 7.0 to 8.0, a surface to volume ratio near unity, and a growth temperature of 25 C. The presence of light and the locus of growth (whether surface or submerged) were not critical factors.

Although good yields of histoplasmin were produced in media containing glucose, far better yields were obtained with either cellobiose, dextrin, starch, or glycogen as the carbohydrate source.

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THE INFLUENCE OF SODIUM ACETATE UPON THE DISSOCIATION OF A STRAIN OF HEMOLYTIC STREPTOCOCCUS

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It is generally recognized that a variety of environmental factors may influence the process of bacterial dissociation. Inasmuch as most of these studies have been conducted with media composed of crude natural materials, it was considered advisable to employ media of known chemical constitution in order to determine more precisely the nutritional factors capable of implementing this variation phenomenon. The inquiry is particularly pertinent in view of the current tendency to explain dissociative behavior, as well as other categories of variation, on a mutation basis. According to Braun (1947), the chief influence of environment is to select out the spontaneously occurring mutants.

The present paper is the first of a series dealing with the use of such "synthetic" media in studies upon bacterial dissociation. The term "synthetic" as used in this work designates media in which a hydrolyzate from purified casein is the only substance the chemical constitution of which is not absolutely established. This study is concerned with the role of sodium acetate in the maintenance of the mucoid character and virulence of a strain of hemolytic streptococcus.

METHODS

Culture. A culture of hemolytic streptococcus, Lancefield group C, Stoddard strain (Mellon and Cooper, 1938), was used throughout the investigation. This culture is mucoid in character and has a high virulence for white mice (MLD = 2 to 6 organisms). As a stock culture, it has been carried in semisolid veal heart infusion agar (0.2 per cent) containing 5 per cent defibrinated rabbit's blood. This medium is adjusted to pH 7.6. The culture has been passed through mice at frequent intervals in order to maintain its virulence.

Determination of virulence. Virulence tests were conducted by preparing varying dilutions of actively growing cultures in veal heart infusion broth and injecting 0.5-ml quantities intraperitoneally into white mice weighing 20 to 25 grams. Each dilution was administered to three mice and the animals were observed for ten days. The MLD was taken as the minimum number of organisms necessary to cause death in two of the three animals in each group.

Special media. The three synthetic media employed are shown in table 1. All experiments were conducted with 5 ml of single strength medium per culture tube.

<div align="center">

TABLE 1

Constituents of media*

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MEDIUM A†			MEDIUM B		
Constituents	Concentration‡		Constituents	Concentration‡	
Casein hydrolyzate§.........	100	ml	Casein hydrolyzate§........	100	ml
KCl....	6	g	Sodium acetate·3H₂O.......	40	g
Na₂HPO₄·12H₂O	2	g	Sodium citrate·2H₂O......	1	g
KH₂PO₄......	5	g	Glucose.	40	g
MgSO₄·7H₂O...............	1	g	K₂HPO₄....................	1	g
Glucose....................	66.5	g	KH₂PO₄..........	1	g
L-Cystine..................	270	mg	L-Cystine.................	200	mg
Uracil.....................	20	mg	L-Asparagine..............	200	mg
Adenine...................	20	mg	Guanine...................	20	mg
Nicotinic acid.......	2	mg	Uracil....................	20	mg
Pyridoxine hydrochloride...	2	mg	Adenine...................	20	mg
Calcium pantothenate	8	mg	Nicotinic acid.............	2	mg
Thiamine hydrochloride.....	2	mg	Pyridoxine hydrochloride....	2	mg
Riboflavin........	1	mg	Calcium pantothenate.	8	mg
L-Tryptophan.....	40	mg	Thiamine hydrochloride.	2	mg
CuSO₄·5H₂O... .... . ; ..	2	mg	Riboflavin.......	1	mg
ZnSO₄·7H₂O.	2	mg	L-Tryptophan.............	40	mg
FeSO₄·7H₂O............ ...	2	mg	Pyridoxal hydrochloride....	2	mg
MnCl₂·2H₂O	0.8	mg	Pyridoxamine dihydrochlo-		
CaCl₂·2H₂O...	20	mg	ride.......	2	mg
Biotin....	0.0004	mg	Xanthine.....	20	mg
			p-Aminobenzoic acid	0.1	mg
Adjust to pH 6.5‖ and auto-			i-Inositol	1	mg
clave			MgSO₄·7H₂O..	400	mg
			FeSO₄·7H₂O....	20	mg
			MnSO₄·2H₂O	12	mg
NaHCO₃¶............. ...	4	g	NaCl..........	20	mg
L-Glutamine¶....	400	mg	CuSO₄·5H₂O...	2	mg
Thioglycolic acid¶......... .	260	mg	ZnSO₄·7H₂O.	2	mg
			CaCl₂·2H₂O.....	20	mg
			Folic acid........	0.001	mg
			Biotin....	0.0004	mg
			Adjust to pH 6.5‖ and		
			autoclave		
			NaHCO₃¶...................	4	g
			L-Glutamine¶..............	400	mg
			Thioglycolic acid¶.........	260	mg
Final pH............................7.5			Final pH............................7.5		

* Medium C was prepared by omitting the following compounds from Medium B:—sodium citrate, L-asparagine, guanine, pyridoxal hydrochloride, pyridoxamine dihydrochloride, xanthine, p-aminobenzoic acid, i-inositol, and folic acid.

† Medium A is essentially that of Bernheimer *et al.* (1942).

‡ Concentration per liter of double strength medium.

§ Casein hydrolyzate, 10 per cent, "vitamin-free," acid-hydrolyzed (SMACO).

‖ The pH was adjusted with 10 per cent sodium hydroxide solution.

¶ Added aseptically to the autoclaved medium.

The glutamine solution was sterilized by filtration through a sintered glass filter. Sodium bicarbonate and thioglycolic acid were sterilized as described by Adams and Roe (1945).

Inoculation procedures. For each experiment a stock culture of maximum virulence was plated on blood agar and incubated 18 to 20 hours at 37 C. A tube of the synthetic medium (table 1) under investigation was inoculated with this culture. After incubation at 37 C for 18 hours, the cells were separated by centrifugation, washed with normal saline, and resuspended in 5 ml of saline. This suspension (0.1 ml) was used to inoculate the original synthetic medium or any modification thereof. After incubation for 4 to 5 hours at 37 C, the growth and pH measurements were made on duplicate cultures. The amount of growth was determined turbidimetrically with an Evelyn photocolorimeter using a 660 filter, and by the plate count method. The pH measurements were made with a Beckman pH meter. After storage overnight at 5 C, 0.2 ml of the culture were used to inoculate a fresh batch of the same medium. This procedure was repeated for every daily serial transfer. Virulence tests were performed on cultures from the final transfer.

In the early experiments, an 18-hour incubation period was employed in the serial transfers. The results obtained with this procedure did not differ from those obtained when the shorter incubation periods were used. The latter procedure has the advantage of avoiding effects due to the lowered pH and to the accumulation of metabolic products found in aging cultures.

EXPERIMENTAL RESULTS

In the original experiments employing synthetic medium A (table 1) a decrease in mucoid character and mouse virulence was noted after daily, serial transfers. Since this culture maintained both of these characteristics on a veal heart infusion blood medium, it is apparent that a constituent (or constituents) present in the natural medium and missing from the synthetic medium A was essential. Experiments to determine the nature of this essential factor (or factors) were initiated.

Our first efforts were directed toward an attempt to prepare a synthetic medium that was comparable to the natural medium in its ability to maintain mucoidness and virulence. It was hoped that a systematic elimination of various components of such a complete synthetic medium would lead to the identification of the essential factor(s). Accordingly, medium A was supplemented with a variety of compounds to yield medium B (table 1). This medium was capable of maintaining the mucoid character and virulence on daily, serial transfer. A systematic elimination of the various components of medium B was undertaken, and it was found that the omission of sodium acetate resulted in a medium which permitted the mucoid to smooth transformation. Sodium acetate was specific in this regard. It should be noted that the original medium employed (medium A) did not contain sodium acetate, thus explaining its inability to maintain the mucoid character.

The omission of the following compounds from medium B affected neither growth nor the ability to maintain a mucoid state: sodium citrate, L-asparagine, guanine, pyridoxal hydrochloride, pyridoxamine dihydrochloride, xanthine, p-aminobenzoic acid, i-inositol, and folic acid. To simplify our procedure,

these constituents were omitted from medium B to yield medium C (table 1). All of the subsequent results reported in this paper were performed with medium C, or with medium C lacking sodium acetate.

On the third to fourth transfer in the medium lacking acetate, transformation from the mucoid into the smooth phase became evident. At the sixth transfer only the smooth phase was present. As the cultural characteristics changed, the morphology of the organism also underwent change. The original mucoid culture composed of encapsulated cocci in very short chains dissociated to a long-chained streptococcus with loss of capsular material. Identical results were obtained in ten experiments. No evidence of any change from the mucoid phase has been noted after 64 daily, serial transfers in either medium C (containing acetate) or in veal heart infusion blood broth.

The virulence of the smooth phase for mice was considerably less than that of the mucoid phase. Thus the MLD of the mucoid phase (grown in veal heart infusion blood broth or in medium C) and the smooth phase (grown in medium C minus acetate) was 5 and 5,000 organisms, respectively.

The stability of the smooth phase was investigated. When subcultured in medium C, no transformation into the mucoid phase was noted after 14 daily, serial transfers. The decreased mouse virulence of the smooth phase was also unaffected by this procedure. In contrast, when passed serially through mice in numbers large enough to cause death, a pure culture of the smooth phase gradually reverted to the mucoid phase. At the tenth serial transfer, only the mucoid phase was evident. The virulence of this culture was identical with that of the original mucoid organism.

As determined both by the plate count and turbidimetric measurements, the omission of acetate from medium C was without effect upon the extent of growth of the mucoid phase during the first few serial transfers. After continued subculture, transformation into the smooth phase occurred. The growth rates of this resulting smooth phase in both acetate-free and acetate-containing media were identical and equal to that of the original mucoid culture. The pH of cultures grown both in the presence and absence of acetate never fell below 7.0. To further eliminate the possibility that the buffering action of acetate was significant in the maintenance of the mucoid phase, cultures were serially transferred in media in which the acetate was substituted by phosphate, citrate, and bicarbonate buffers. The mucoid phase could not be maintained in the presence of these buffers, thus demonstrating that the activity of acetate was not due to its buffering capacity.

DISCUSSION

The application of "synthetic" media to studies upon bacterial dissociation possesses a twofold advantage. First, the reproducibility of the medium can be maintained within rather narrow limits. This is of particular importance since there is formidable evidence that variations in environment can markedly influence the process of bacterial dissociation. The extreme consistency of the results reported here bears witness to the reproducibility of our environmental

conditions. The past experience of this laboratory in studies of this type has been entirely with media composed of crude, natural materials and a not infrequent inability to reproduce certain results has been noted. It is conceivable that variations in the composition of the natural materials contained in these media could account for some of these anomalous results.

In the second place, a medium of known chemical constitution is essential in studies designed to determine the role of individual nutrilites upon bacterial dissociation. The flexibility of such media in regard to the ease with which the individual components may be varied is evident.

The present experiments have demonstrated that the elimination of sodium acetate from a "synthetic" medium determines the transformation from the mucoid to the smooth growth phase. It should be noted that as long as the mucoid phase is able to grow without acetate, the absence of the latter affects neither the growth rate of the organism nor the final pH of the culture medium. The M to S phase change, therefore, is due primarily to the absence of sodium acetate and is not the result of secondary influences upon rate of growth or degree of acidity. This point requires emphasis since changes in the pH of the medium, and bacteriostatic changes particularly, are well known to affect the incidence of bacterial dissociation. The fact that the M phase is replaced by the S phase in the absence of acetate is not interpreted as a bacteriostatic or bactericidal effect, but rather a change incident to a nutritional deficiency.

With regard to the mechanism of action of the sodium acetate, we have adopted the tentative view that this compound functions not as a growth factor but as a precursor in the biosynthesis of some higher molecular weight compound(s) which is essential—perhaps, as a structural component of the capsular material—for the maintenance of the mucoid state. Apparently an exogenous source of acetate is required for this function. In the absence of added acetate, the transformation to a smooth phase, which is stable even in the presence of acetate, occurs.

The alternate view, namely, that acetate is essential for the growth of the mucoid and not the smooth phase, is difficult to substantiate, since transformation into the smooth phase occurs following continued subculture in the absence of acetate. At least for the first few serial transfers into a medium lacking acetate (before the M to S change is recognizable) no bacteriostatic effect is noted.

The precise nature of the postulated compound into which acetate is presumed to be converted is unknown. There is ample evidence that acetate may serve in the biosynthesis of a variety of tissue constituents, such as glycogen, uric acid, fatty acids, cholesterol, dicarboxylic amino acids, and protoporphyrin (Bloch, 1947). Guirard et al. (1946) have reported on the probable role of acetate as a precursor of various lipoidal materials in bacterial metabolism.

Preliminary experiments have indicated that lipids may replace acetate for the maintenance of the mucoid character of our strain of hemolytic streptococcus.

The relationship between mucoid character and virulence is not clear. In the group C organism employed in this study, maximum virulence is characteristic

only of the mucoid state. The conditioning influence of the acetate on the mucoid state in these experiments tends to emphasize the likelihood that mucoid nature and virulence do not represent linked characters, but rather an association of characters. It is interesting to note in this connection that our experience with certain strains of group A streptococci has indicated that an exogenous source of acetate is not necessary for either the growth or the maintenance of mucoid character. Although the majority of group A strains are highly virulent in the mucoid phase, it has long been known that exceptions occur. In the case of group A streptococci, it is well known that virulence is dependent upon the presence of the M antigen, and the presence of this antigen gives rise to the so-called "matt" appearance that is characteristic of the colonies produced by virulent, but nonmucoid, strains of group A streptococci.

The current genetic appraisal of this M to S transformation would probably say that we are dealing with a very low frequency of mutation rate on the part of the mucoid phase, which phase has a suppressive effect on the appearance of the resulting S mutant. This suppressive effect would be abrogated by the absence of acetate, which would select out the occasional mutant S cell by depriving the M cells of a chance to grow.

This interpretation, however, fails to accord with the results of experiments designed to test its validity. Thus two single S phase cells were mixed in acetate-containing broth containing 5,000,000 M phase cells. After 18 hours' incubation at 37 C, equal numbers of M and S forms were present.

Furthermore, after 60 serial transfers of the M phase on blood agar had shown no S phase colonies, 12 isolated M colonies from a single plate were selected at random for test of the frequency of the M to S transformation. Transfers were made from each of six colonies into its own tube of acetate broth; and six colonies were likewise transferred into the same synthetic medium without acetate. After the fifth serial transfer in the latter medium, all six M phase cultures went over to the S phase, with only occasional mucoid colonies appearing. At this time all six M phase cultures in acetate broth remained mucoid, no S colonies appearing.

In other words, there is not the slightest evidence of a suppressive effect of M over S; in fact, the phenomenon of "population dynamics" appears to be in reverse. As to frequency, the transformation occurred in 100 per cent of the colonies tested. Thus, it seems permissible to infer that the burden of proof in this instance is shifted to the opponents of the theory of adaptive modification by specific environments.

SUMMARY

It has been demonstrated that the presence of sodium acetate in a synthetic medium is necessary in order to maintain the mucoid character and virulence of a strain of group C hemolytic streptococcus upon repeated daily subculture. The elimination of acetate permits the transformation from the virulent mucoid phase to a relatively avirulent smooth phase. The smooth phase does not revert to the mucoid state *in vitro* in the presence of acetate, but does completely

revert after ten passages in mice.   This phase change has been shown to be due primarily to the absence of sodium acetate and is not the result of secondary influences upon rate of growth or degree of acidity.

All the evidence to date points to the likelihood that the transformation is not the result of a spontaneous mutation, but is an adaptive response to the absence of a nutrilite so vital to the mucoid phase that this phase may be said to have no existence in its absence.

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STUDIES ON HEAT RESISTANCE

I. Increasing Resistance to Heat of Bacterial Spores by Selection

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Although many data may be found in the literature concerning the resistance of bacterial spores to heat, little work has been published on the specific phase of thermal resistance studies pertaining to an increased resistance under certain conditions of natural or artificial selection.

Bigelow and Esty (1920), by repeated culturing of survivors from under-sterilized spore suspensions, were able to increase the resistance of the spores of certain obligate thermophiles from 12 to 23 minutes at 120 C. Magoon (1926) reported an increase in thermal resistance of the spores of *Bacillus mycoides* from 2 minutes to 44 minutes at 100 C, and O. B. Williams (1929) observed an increase in the resistance of spores of *Bacillus subtilis* from 38 to 54 minutes at 95 C, each after the selection of survivors of heated suspensions.

But other workers have been only partially successful, and many entirely unsuccessful, in selectively increasing bacterial resistance to heat. Thus, for example, F. T. Williams (1936), using single cell cultures of *Bacillus mycoides*, *Bacillus subtilis*, and others, was able to increase the resistance of *Bacillus mycoides* only; his results showed an increase from 10 to 25 minutes at 100 C after 10 selective transfers, but he reasoned that the clumping tendency of this organism may have been a factor in the enhanced resistance. Sommer (1930) reported that *Clostridium botulinum* exhibited no tendency to increase in resistance to heat after careful selection, and Morrison and Rettger (1930), working with two aerobic, mesophilic, sporeformers from spoiled canned milk, found the heat resistance of these organisms unchanged after attempting for 6 months to alter their resistance by selection. The evidence for an increase in thermal resistance by selection has been severely criticized by Rahn (1932, 1943, 1945), who believes that the last survivors are no more resistant than the average, having survived the heating period merely by chance.

It has been the purpose of this investigation to re-examine the question of whether the thermal resistance of the spores of bacteria may be increased by the selection of survivors of exposure to heat for a time sufficient to destroy almost all of the spores present.

EXPERIMENTAL METHODS AND RESULTS

Culture used. Single colony isolations were made from platings of *Bacillus globigii*,[1] a nonspreading, aerobic, mesophilic, sporeforming rod that produces on

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[1] *Bacillus globigii* was from the stock culture collection, Department of Bacteriology, The University of Texas. This organism is described in the sixth edition of *Bergey's Manual of Determinative Bacteriology* as identical with, or a variant of, *Bacillus subtilis*.

nutrient agar and brain-heart infusion agar media distinctive deep-orange-pigmented colonies at either 37 C or at room temperature. This organism was chosen because of the ease with which it may be distinguished from contaminants, as well as for its nonspreading characteristic. The single colony isolates were maintained on nutrient agar slants and used as source cultures when required.

Methods of preparing spore suspensions. For initial thermal resistance tests Kolle flasks of nutrient agar (Difco), pH 7.0, were inoculated with 1-ml aliquots of a suspension of the test organism in sterile water; incubation at 37 C for 5 days resulted in cultures containing approximately 95 per cent spores. The growth was harvested from the surface of the medium, washed twice with sterile distilled water by centrifugation, suspended in sterile M/15 double sodium phosphate buffer, pH 7.0, and filtered through double sheets of sterile coarse filter paper to remove clumps of spores. The spore suspensions were counted by means of the Petroff-Hauser chamber, and these stock suspensions were stored in the refrigerator for use in later experiments.

Method of heating spore suspensions. In the actual determination of the thermal death time of the spores of *Bacillus globigii*, stock spore suspension samples were diluted with sterile M/15 double phosphate buffer to give test suspensions of 50 million spores per ml. Two ml of the test suspension employed, a total of 100 million spores, were pipetted into each of several pyrex glass tubes, 9 mm in diameter and approximately 12.5 cm in length, which had been cleaned in potassium dichromate, sulfuric acid mixture and rinsed in tap water and distilled water before being sterilized in the hot air oven. The tubed suspensions were sealed with an oxygen torch and immersed completely in a water bath held constant at 98.5 C (boiling); specially designed tube holders prepared from heavy wire strands, squares of screen wire, and metal loops made possible the immersion of the tubes in groups of five. Six of these holders were employed, permitting a total of 30 tubes to be heated simultaneously. After allowing a 1-minute interval for the diffusion of heat throughout the suspension, groups of 5 tubes were withdrawn from the boiling water at the desired time intervals and plunged into cold water.

The tips of the sealed tubes were broken aseptically by the use of a bar devised by A. C. Richardson and described by O. B. Williams (1929), and the spore suspensions were inoculated into sterile plates, one tube per plate. Brain-heart infusion medium (Difco), pH 7.0, to which had been added 2 per cent agar and 0.1 per cent soluble starch, was poured over the inocula and allowed to solidify, and the plates were incubated at 37 C. Plate count results were read at intervals up to 10 days. In every case at least 5 tubes were prepared for each time interval of heating, and in many cases, in the critical temperature range, 10 or 15 spores suspensions were withdrawn at each interval. The importance of using a series of suspensions has been emphasized most recently by Baker and McClung (1939) in a study of "skips" in thermal death time determinations.

Selection of survivors. In an attempt to increase the resistance of the spores of *Bacillus globigii* to heat, survivors of death time determinations were chosen, when possible, from plates bearing from 30 to 100 colonies each; no attempt was

TABLE 1

Thermal death time determinations on spores of Bacillus globigii

SELECTION SERIES	TIME IN MINUTES AT 98.5 C								
	6	8	10	12	14	16	18	20	22
Initial test	TMC	11*	1	0	0	0			
1		6*	0	0	0	0	0		
2		147	27*	2	0	0	0		
3		TMC	324	22*	4	2	0	0	
4			490	29*	6	0	0	0	
5				63*	6	1	0	0	0
6				TMC	59*	9	2	0	0
7				TMC	27*	6	1	0	0
8				120*	3	0	0	0	0
9				TMC	457	14*	0	0	0
10				TMC	209	22	0	0	0

Figures represent the average number of colonies per plate from 5 or more plates at each time interval.

TMC = too many colonies to count.

* Representatives from these colonies were picked as inoculum for another spore harvest.
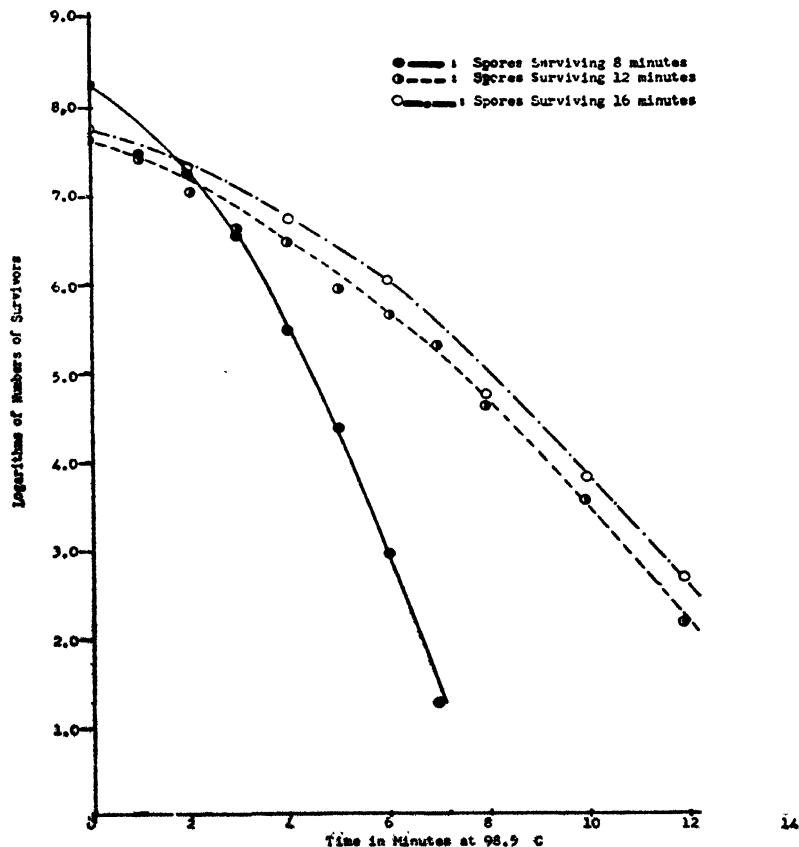
Figure 1. Heat destruction curves, *Bacillus globigii*.

made to select the very last survivor of a test run.   The colonies were picked with a loop needle to prepare a suspension of survivors in sterile water, and this suspension was used to inoculate Kolle flasks of nutrient agar medium for another spore crop; these spores were harvested, washed, filtered, heated, and plated, and the survivors were again picked for a continuation of the selection process. The results of end point determinations are presented in table 1.

Determination of thermal death time by destruction curve.   When it became apparent from inspection of the results of death time determinations that a marked increase in resistance to heat had occurred in the spores of *Bacillus globigii*, it appeared desirable to confirm these results by plotting destruction curves based on the number of spores surviving per unit of time of heating at 98.5 C.   It was reasoned that the increase in resistance would manifest itself in the slope of the curves.   Toward this end three stock spore suspensions were chosen, one of which had given an average of 11 colonies per plate at 8 minutes of heating, the second an average of 22 colonies per plate at 12 minutes, and the third 22 colonies per plate at 16 minutes.   The heating technique described earlier was employed, except that only 1 ml of spore suspension, 50 million spores, was heated in each tube; the 1-ml suspensions were poured into sterile water blanks and serial dilutions were plated.

The curves resulting from plotting the logarithms of the number of survivors against time, presented in figure 1, show a distinct difference in destruction rate between the low-resistant and the high-resistant spores.

DISCUSSION

Many workers, including Bigelow and Esty (1920) and Sommer (1930), have observed that the bacterial cells in a given culture vary markedly in resistance to heat; Baker and McClung (1939) found that different suspensions of the same organism, heated at the same temperature, exhibit differences in the rate of death as judged by death time curves, and Knaysi (1948) has stated that the resistance of spores in a given bacterial species is not a fixed value.   Confirmation of this evidence of a gradation of resistance within a spore population has been given by Yesair and Cameron (1936), who, by centrifuging spore suspensions and withdrawing samples at intervals for heat resistance tests, were able to correlate length of time of centrifugation with resistance to heat.   From these experiments they concluded that perhaps either the specific gravity or the size of the spores could be correlated with thermal resistance.

These observations by other workers and the experimental results reported here indicate that there exists a graded resistance to heat among the spores of a population.   By the selection of survivors of heated spore suspensions both a qualitative and a quantitative increase in resistance has been effected, as is evidenced by end point determinations (table 1), and by destruction curves (figure 1).

SUMMARY

Heat resistance studies on the spores of *Bacillus globigii* have been presented. The spores of this organism, derived from single colony isolates, exhibited an

increase in resistance to heat, as judged by thermal death time determinations and by destruction curves when survivors of heated suspensions were selected for further heating tests.

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STUDIES ON HEAT RESISTANCE

II. Comparison of Resistance to Heat with Resistance to Disinfectants[1]

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The literature pertaining to studies on the thermal resistance of bacterial spores reveals few successful attempts to increase the resistance of these forms by selective cultivation of survivors of heated spore suspensions. Among the workers presenting evidence for such an increase are Bigelow and Esty (1920), Magoon (1926), O. B. Williams (1929), F. T. Williams (1936), and Davis and Williams (1948).

The authors are unaware of any published data concerning a correlation of the increased thermal resistance of bacterial spores with increased resistance to chemical agents. From this standpoint it appeared desirable to ascertain whether the spores of a culture whose resistance to heat had been enhanced by repeated subculturing of survivors would exhibit a greater resistance to certain commonly employed bacteriostatic and bactericidal agents than do the spores of the parent culture from which these resistant forms were derived. Comparative studies are presented on the bacteriostatic action of mercuric chloride, gentian violet, and streptomycin, and the sporicidal action of phenol and iodine for a stock strain of *Bacillus globigii* and a heat-resistant variant developed from it.

EXPERIMENTAL METHODS AND RESULTS

Bacterial cultures. Two stock spore suspensions of *Bacillus globigii* derived originally from a single colony isolate were employed throughout this investigation. The spores of the mother culture were found by thermal death time determinations and heat destruction curves to survive 8 minutes' exposure at 98.5 C, whereas the spores of the heat-resistant strain derived from the mother culture (Davis and Williams, 1948) survived for 12 minutes. Heat destruction curves for the two spore suspensions are presented in figure 1.

Comparative resistance to the bacteriostatic agents, mercuric chloride, gentian violet, and streptomycin. One-ml aliquots of each stock spore suspension containing 50 million spores per ml were plated in parallel over a wide dilution range in nutrient agar (Difco), pH 7.0, to which had been added appropriate concentrations of one of the following agents, mercuric chloride (Merck), gentian violet (National Aniline and Chemical Co.), and streptomycin sulfate (Abbott). The poured plates were incubated for 3 days at 37 C, and the plate counts showed

the number of individuals in the two populations resistant to the bacteriostatic action of the inhibiting agents. The plots of the survivor curves presented in figures 2, 3, and 4 show that the heat-resistant spores do not differ from the parent culture in resistance to 3 types of bacteriostatic agents.

Comparative resistance to the sporicidal action of phenol and iodine. Flasks
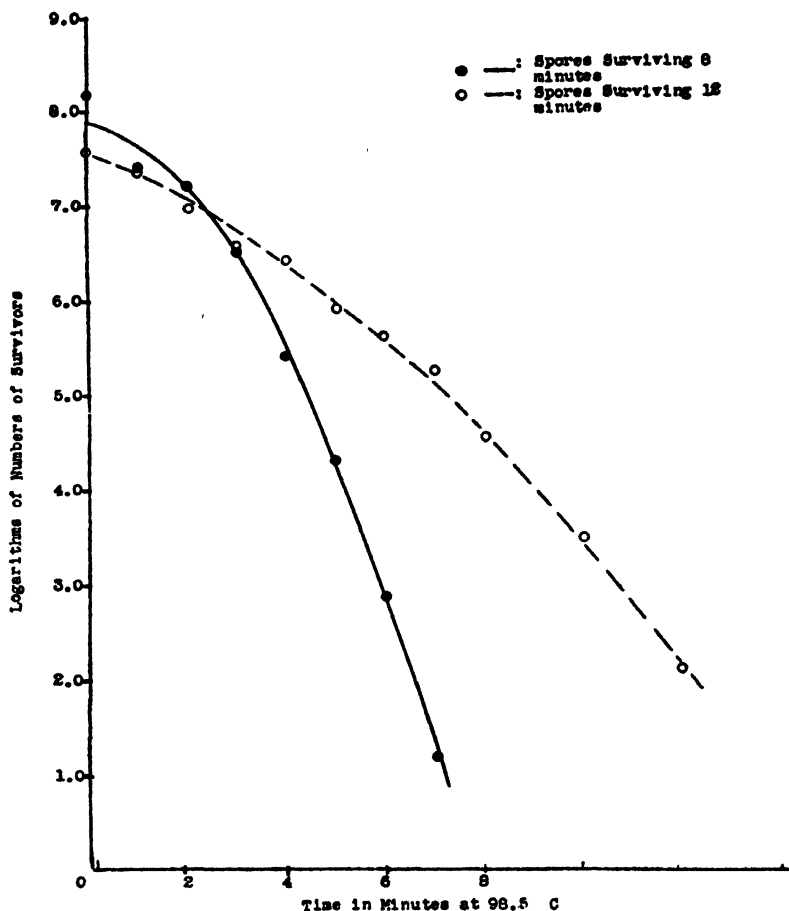
Figure 1. Heat survivor curves, *Bacillus globigii.*

containing 1 liter of a 5 per cent solution of phenol (Merck) in sterile water were inoculated in parallel with samples of the two stock spore suspensions of *Bacillus globigii* to give a spore concentration of 200,000 per ml. The flasks were incubated at room temperature; at approximately 24-hour intervals 10-ml aliquots were withdrawn and pipetted to sterile 90-ml water blanks containing 1 gram of activated charcoal (norite) to stop the action of the phenol; appropriate dilutions were plated with nutrient agar (Difco), pH 7.0, to which had been

added 0.1 per cent norite.　The importance of using an antidote in disinfection studies on phenol has been discussed by Flett, Haring, Guiteras, and Shapiro (1945).　Plate count results were read after 3 days' incubation at 37 C, and survivor curves were plotted (figure 5).　It is evident that the strain of enhanced heat resistance is also more resistant to phenol.
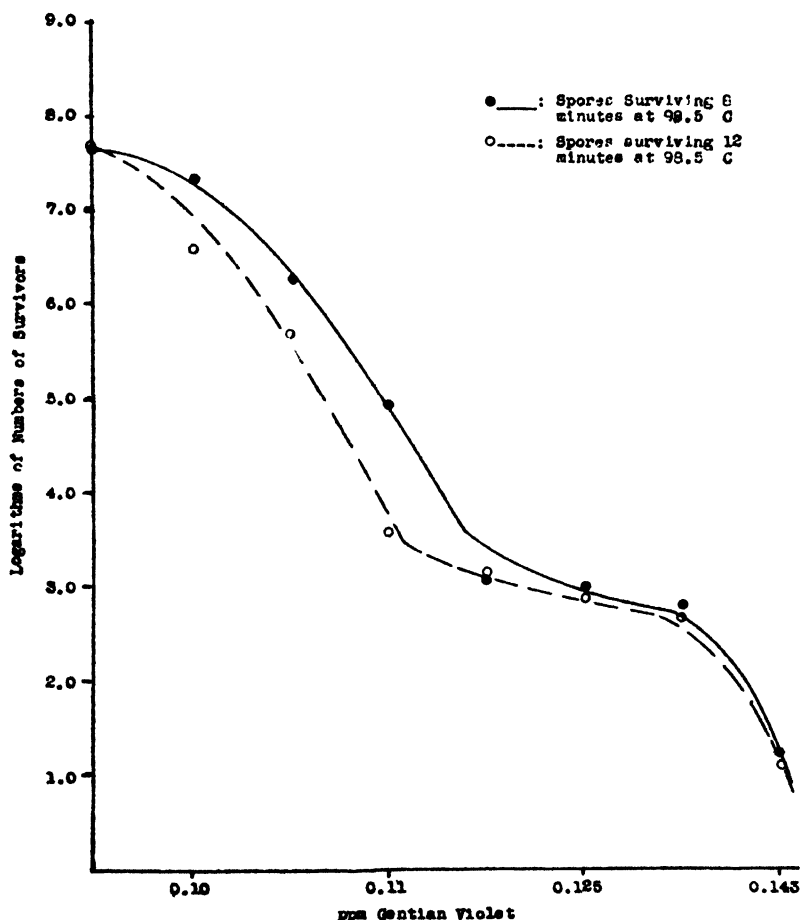
Figure 2. Survivor curves on *Bacillus globigii* (gentian violet).

Employing a modification of the method of Wyss and Strandskov (1945), a 2 per cent alcoholic solution of resublimed iodine (Baker) was added to flasks of sterile M/10 phosphate buffer, pH 7.0, to give an iodine concentration of 200 ppm. At zero time the two spore suspensions were added to parallel flasks to give a concentration of 200,000 spores per ml.　At measured time intervals, samples were withdrawn and pipetted immediately to sterile tubes containing a drop of a boiled, 10 per cent solution of sodium sulfite to inactivate the iodine.　Appro-
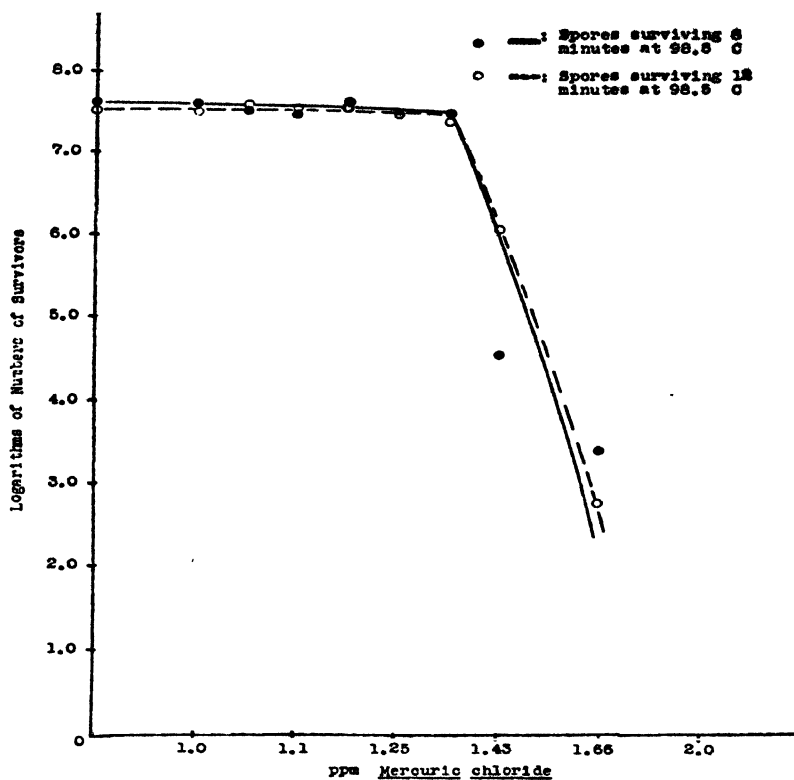
Figure 3. Survivor curves on *Bacillus globigii* (mercuric chloride).
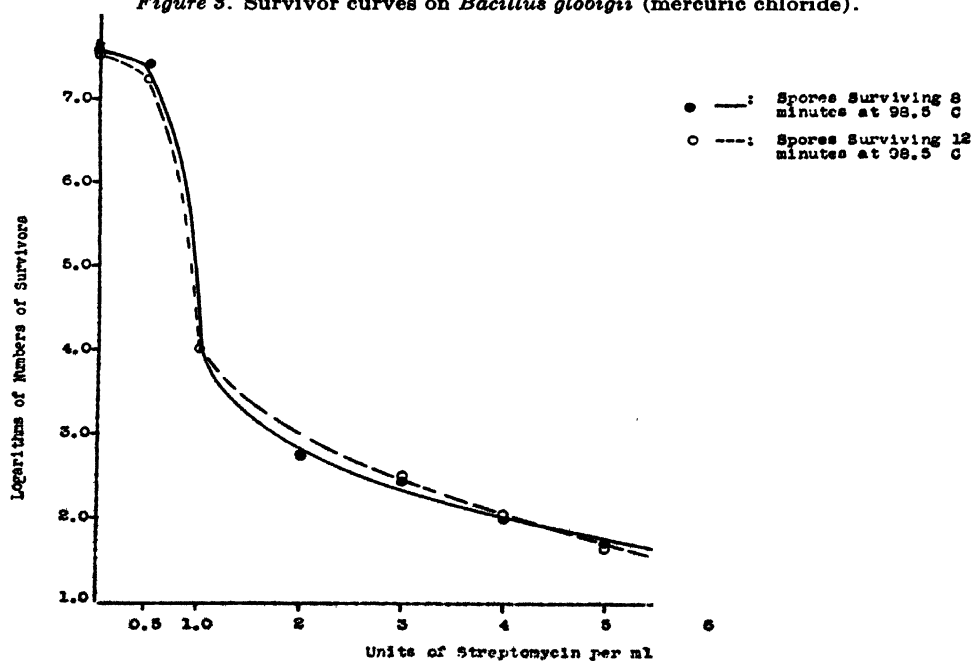
Figure. 4. Survivor curves on *Bacillus globigii* (streptomycin).
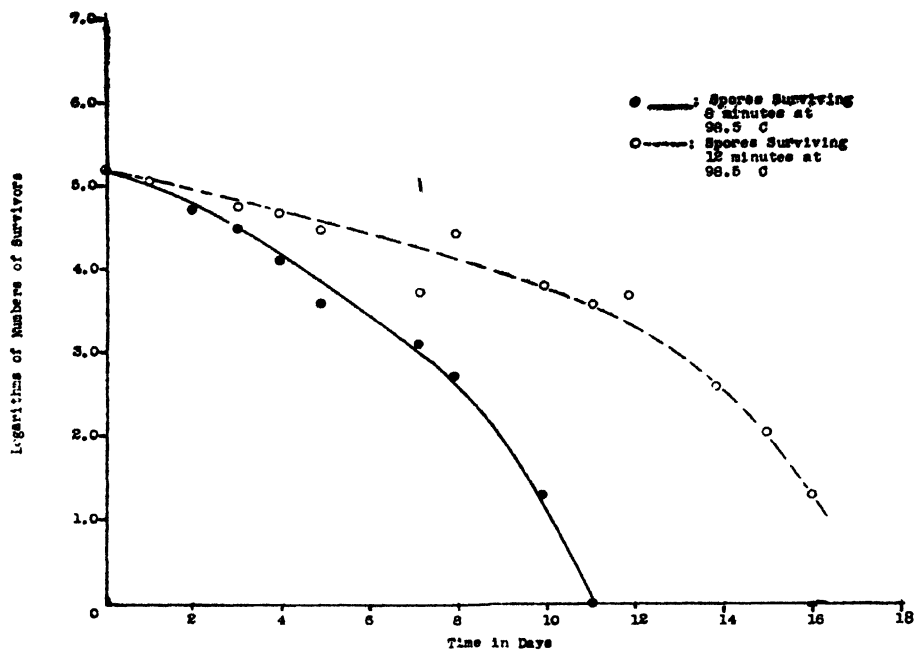
Figure 5. Survivor curves on *Bacillus globigii* (phenol 5 per cent).
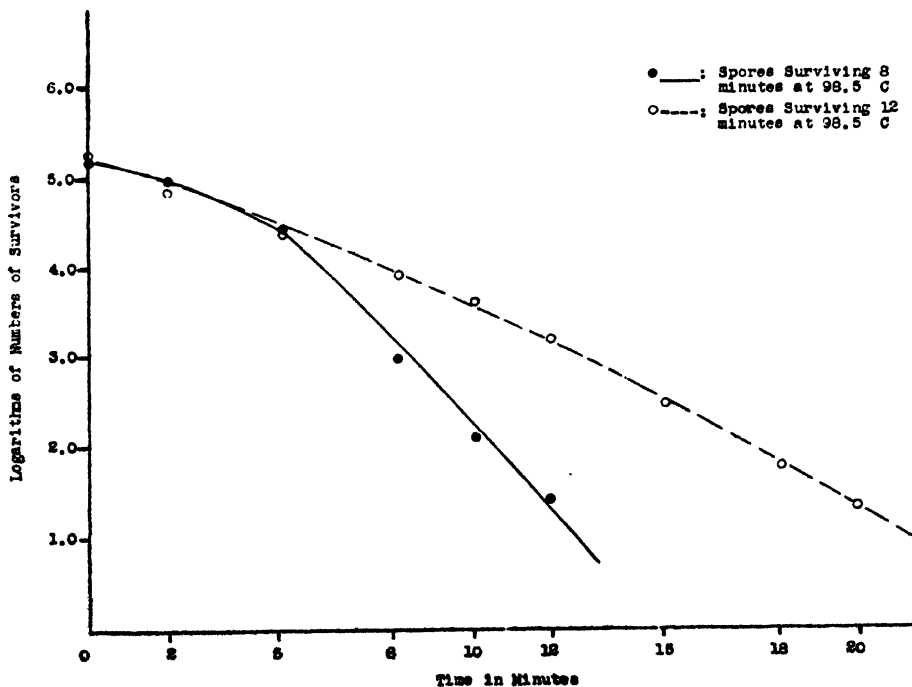
Figure 6. Survivor curves on *Bacillus globigii* (iodine 200 ppm).

priate dilutions of this neutralized suspension were plated in nutrient agar (Difco), pH 7.0. Plates were incubated at 37 C and read after 3 days. The resulting survivor curves presented in figure 6 show that the heat-resistant spores were also resistant to iodine.

DISCUSSION

Studies on the mechanism of action of the inhibition displayed by the bacteriostatic and bactericidal agents employed in these experiments have been discussed by Rahn (1945), McCulloch (1936), Wyss (1948), and Waksman (1947).

It appears from the inspection of survivor curves that germination and colony development from the spores of the two suspensions are approximately equal in the presence of three bacteriostatic agents whose modes of action are widely different. This suggests that the mechanism of heat resistance of the spore may not be reflected in observable changes in the vegetative cell and correlates with the observation that the vegetative cells of the heat-resistant subculture are no more heat resistant than the vegetative cells of the parent strain (unpublished results, Davis and Williams). Furthermore the two spore suspensions differ markedly in resistance to the killing action of phenol and iodine. The exact mode of action on the bacterial cell of these two agents has not been determined, although concepts of their disinfectant action have been reviewed by Wyss (1948). Further investigation of the correlation of thermal resistance and resistance to chemical agents may well give an insight into the basis of heat resistance in spores and permit us to discard at least some of the explanatory theories that have been advanced.

SUMMARY

The spores of a culture of *Bacillus globigii* and of a heat-resistant variant derived from it have been tested for resistance to bacteriostatic and bactericidal chemical agents.

Both cultures exhibited approximately equal susceptibility to the bacteriostatic action of gentian violet, mercuric chloride, and streptomycin.

The spores of greater resistance to heat appeared to possess enhanced resistance to the killing action of iodine and phenol.

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NEW PREPARATION TECHNIQUES FOR THE ELECTRON MICROSCOPY OF BACTERIA

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In the early application of the electron microscope to the examination of bacteria, the method of preparation involved the preparation of a distilled water suspension of the organisms and the evaporation of a drop of the suspension on a suitable supporting membrane. Although it was recognized that such treatment probably gave rise to considerable artifacts, this procedure nevertheless was used in most of the published work (Mudd and Anderson, 1944). Recently some of the artifacts introduced by this method of preparation have been investigated and a new method has been suggested (Hillier and Baker, 1946; Edwards and Wyckoff, 1947). This method involves the intact removal of the surface layer of organisms from a culture of a solid medium by means of a thin plastic membrane formed in place on the culture by the evaporation of a suitable solution. Further work on this technique has shown that although it is ideal for the examination of very young cultures of certain strains of organisms, it has numerous serious limitations that will prevent it from being generally useful. For instance, even for those types of organisms for which it can be used, it is not successful if the growing conditions are not "quite right." More serious, however, is that the physical organization of the culture is maintained only in the outermost surface of the growth. Thus, unless the preparations use single-layer growths of strong cells, the resulting specimen is confused by material pulled from inner layers in disorganized masses. This last difficulty was particularly troublesome in recent attempts by one of us to follow, by this technique, the infection of *Escherichia coli* by T₂ bacteriophage.

As a result of these difficulties a new technique has been developed that appears to have many advantages. Basically, it involves growing the organisms directly on the thin supporting membrane of the electron microscope specimen mount in such a way that any direct contact with the organism is avoided throughout the procedure. The only disturbance of the organisms that can introduce artifacts is a final desiccation. For organisms grown on ordinary media, such artifacts have not been observed. At the time of writing, this technique has proved uniformly successful in the preparation of specimens of *Bacillus mycoides*, *Bacillus megatherium*, and *Escherichia coli* grown under a number of different conditions and subsequently treated in several different ways. It would appear in principle at least that the technique should be successful for all organisms that produce surface colonies.

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TECHNIQUE FOR SOLID MEDIA

Figure 1 consists of a series of drawings showing diagrammatically the various steps in the new technique when it is used for a solid medium. Thinly poured agar plates were used in this work. In the first operation (a) the agar plate is flooded with sterile distilled water from a pipette with a fast delivery rate or, preferably, from a flask or a bottle. As soon as the entire surface of the plate is covered, a drop of collodion solution (0.5 to 1.0 per cent in amyl acetate) is dropped on the surface and allowed to spread (b). It is necessary to flood the surface and form the collodion membrane as rapidly as possible in order to mini-
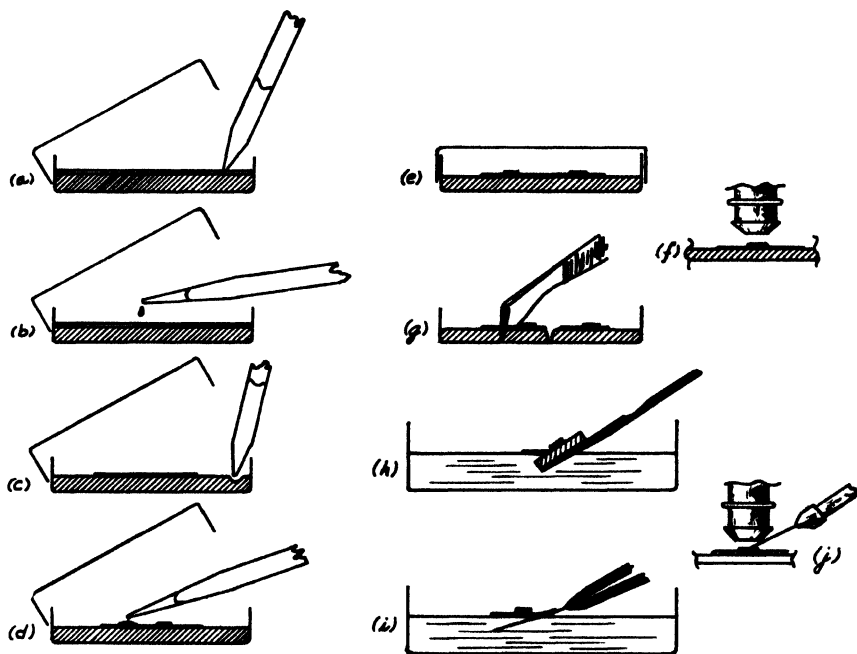
Figure 1. Explanation in text.

mize the time allowed for the agar material to go into solution. Too much agar material in the water will prevent the collodion solution from spreading sufficiently and will result in a thick membrane.[2] After the film is spread, sufficient ventilation of the surface is allowed to permit the solvent to evaporate. No traces of the solvent must be left as they tend to inhibit growth. When the solvent is completely gone, the water is pipetted from under the membrane, allowing it to settle in close contact with the agar (c). In the last stages of this operation, the plate is tilted in order to allow the water to drain completely from

[2] More recently, in using this technique, Mr. Andrew Smith of the University of Pennsylvania has found that the collodion solution spreads much more satisfactorily if the surface of the agar is washed twice with sterile distilled water before being flooded with the water on with the collodion is spread.

between the membrane and the agar.   At this point, the membrane should be checked for thickness by observing the reflection from it of light from a bright extended source.   A sufficiently thin membrane should be completely invisible except for a thickening at its edges.   A membrane that shows a definite sheen will be too thick.

Sufficiently thin portions of the membrane are inoculated with clean distilled water or saline suspensions of the selected organism, a small drop being placed on the membrane from a capillary pipette and immediately withdrawn (d).   If the membrane is wet by the suspension, this procedure will leave a small disk of liquid- a portion of a millimeter thick and 1 to 2 millimeters in diameter.   A direct correlation has been observed between wetting of the membrane and ability of the organisms to grow.   In later work, it was found that suspensions that do not wet the collodion membrane may be made to do so by reducing their surface tension with as little as 0.05 per cent of tryptone.   The tryptone added does not seem to produce an undesirable effect on the cleanliness of the background and is probably removed by dialysis in a later step when the film is floated.   The preparation is then incubated (e); the cover may be left partly open so that the remaining moisture of the inoculum can dry rapidly if it is deemed desirable to prevent the growing cells from moving.

The condition of the inoculum and the growth of the organisms can be followed by examination with a light microscope, using a high-powered dry objective (f). It is desirable that the objective be warmed by keeping it in the incubator if condensation on the first surface is to be avoided.

Since by this technique all new cells and all other products of the growth are retained on the specimen, the period of incubation for normal cells is very short – usually between 1 and 3 hours.   In fact, once established, the growth rapidly becomes too thick for electron microscope examination.

When the growth has reached a desired stage, as determined by light microscope observation, a selected area of the agar and membrane is cut out (g).   In this operation it is desirable to cut the membrane by a sharp scalpel pushing perpendicularly into the agar some distance (8 to 10 mm) from the area selected for examination.   The slab of agar bearing the membrane on its upper side is then lifted from the petri dish and slid under a clean surface of distilled water (h).   The section of membrane floats easily on the surface of the water, bearing the growth on its upper side.   The membrane is then picked up on a sufficiently large, carefully cleaned piece of 200-mesh screen, which is held in fine forceps and raised through the water surface under the floating membrane (i).   The screen is held at a slight angle to the surface so that the membrane is kept smooth and taut by that part of it that remains floating.   As soon as the membrane is lifted on the screen, the water remaining in the openings is absorbed by a hard filter paper so that the preparation dries very rapidly.

Since the growth is on the side of the membrane not in contact with the screen, it can be shadow-cast or treated in other ways with little difficulty.

In this form the specimen can be examined conveniently under a light microscope and suitable individual cells or colonies selected for examination in the
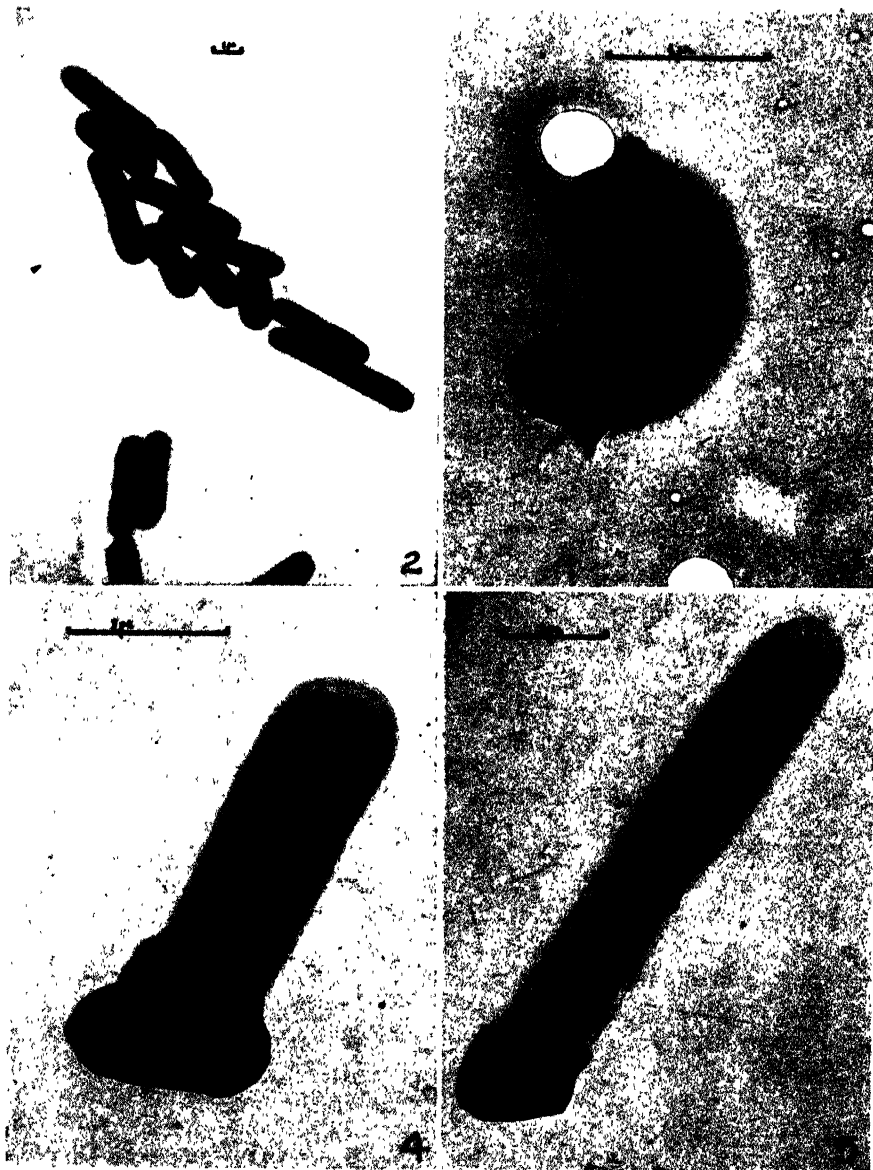
Figure 2. Small colony of Escherichia coli grown from single cell on thin collodion backed by nutrient agar (3 hours at 37 C).   Magnification 3,500 ×.
    Figures 3 and 4. Germination of spores of Bacillus mycoides on thin collodion backed by nutrient agar (2½ hours at 37 C).   Magnification 21,000 ×.
    Figure 5 Another preparation of B. mycoides after 3¼ hours at 37 C.   Magnification 13,000 ×.

electron microscope.   Their general location can be marked by a sharp needle (j) and an area cut or punched out to fit the electron microscope specimen holder.
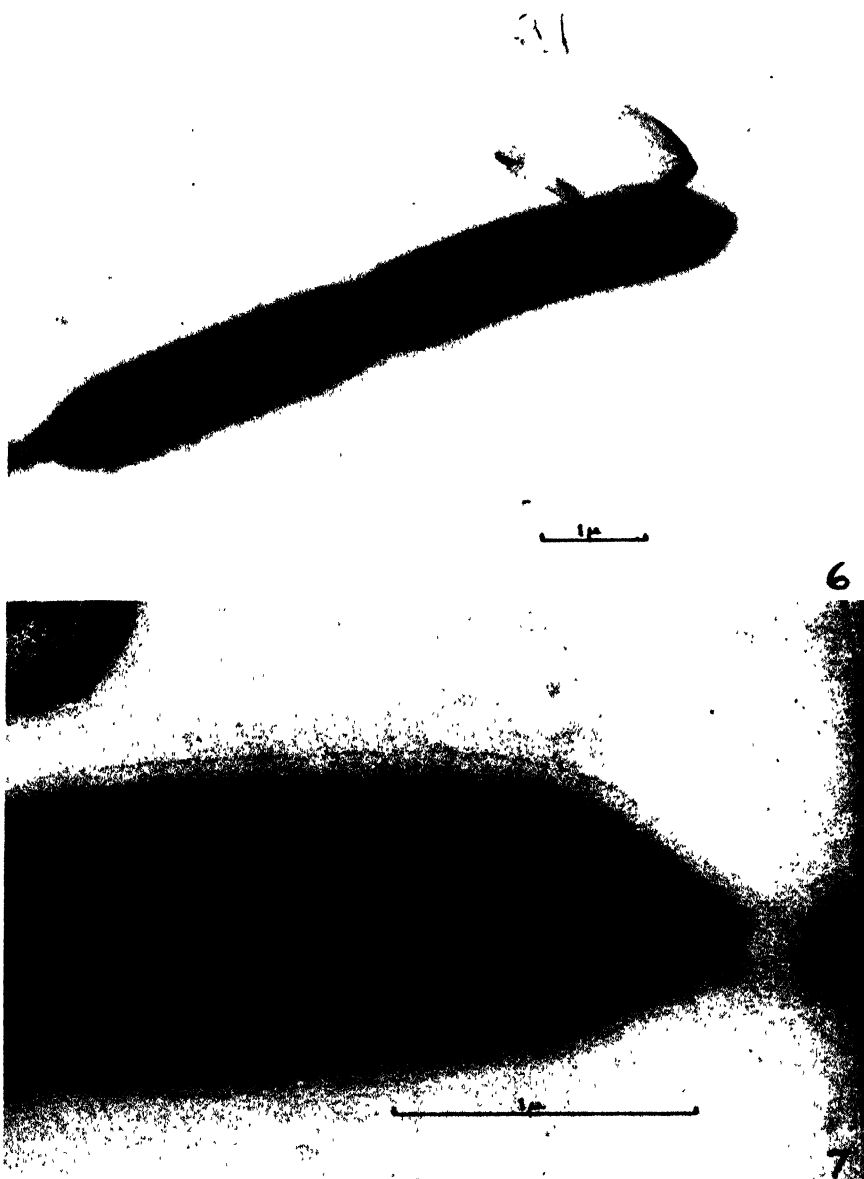
Figure 6. Bacillus megatherium grown on thin colloidion floating on broth (5 hours at 36 C). Magnification 13,000 ×.

Figure 7. Enlargement of another field of same specimen showing the preservation of delicate structures at the point of division. Magnification 40,000 ×.

Figures 2 to 5 are electron micrographs of a small single colony of *E. coli* and single cells of *B. mycoides* grown by this technique. In all such preparations the specimens have been singularly free of extraneous material and desiccation arti-

facts.   Even the usual shrinkage of the protoplasm from the cell wall seems to be minimized.   Of greater importance, however, is the lack of disturbance of sensitive structures such as flagella or the forms found at the point of division of the cells (figure 7).

<center>TECHNIQUE FOR LIQUID MEDIA</center>

For liquid nutrient media, the technique (with some obvious modifications) can also be used for aerobic bacteria.   In this case the membrane is formed on sterile distilled water in a petri dish.   After evaporation of the solvent, the water is pipetted off and replaced by the liquid medium.   In this process, the membrane is deposited on the bottom of the dish and then refloated when the liquid medium is introduced.   Since sterile handling is essential and since large quantities of the medium are not needed for the small numbers of organisms grown by this technique, it is desirable to use petri dishes filled to a depth of 4 to 5 millimeters.   The inoculation of the membrane, the incubation, and final mounting are similar to the preceding method with the exception that the medium is replaced by two changes of water before picking up the membrane on the screen. Figures 6 and 7 are electron micrographs of germinating cells of *B. mega herium* grown by this technique.

<center>DISCUSSION AND CONCLUSIONS</center>

The technique described in the foregoing has achieved for three organisms some definite advantages over the earlier techniques used for the preparation of bacteria for the electron microscope.   In this method the organisms are given a minimum of undesirable treatment.   Although they are grown with a thin (100 to 200 A) membrane separating them from the nutrient medium, no harmful effects can be detected either from light microscope observation of the growing colonies or from the electron microscope results. ' During the remainder of the procedure, the organisms are given no unusual physical or chemical treatment with the possible exception of the short interval when the membrane is in contact with water.   Even in this case the organisms are separated from the water by the membrane, and hence any interaction will be the result of diffusion- a relatively slow process.   In no part of the procedure are the new cells subjected to the effects of mixing in water.   When the organisms are grown under the near-dry conditions described, the surface film of liquid that surrounds them is everywhere nearly parallel to the contour of the growth so that surface tension effects are minimized.   However, if more moisture is present, a droplet may form on the inoculated area.   If this is the case—it can be identified by light microscope examination there may be a rearrangement caused by surface tension during drying.   This last case is to be avoided, of course, if the exact physical relationships in the growth are to be preserved.

Although the technique has been tried for only three species of organisms, those attempts have been uniformly successful.   There seems to be no fundamental reason why it should not be equally successful for a large number of other species.

Figure 8. E. coli strain B grown on thin collodion backed by nutrient agar, inoculated after 1¾ hours at 37 C with T₂ bacteriophage, and then incubated 20 hours at 37 C. Large numbers of bacteriophage particles, various types of cell debris, and a few unlysed cells are all retained near their original positions. Magnification 12,000 ×.

Since in this technique the physical relationships between the organisms are preserved, it is particularly useful for studying the morphological changes that occur as the organisms multiply. The method should also be useful for studying

the effects on bacteria of various chemical agents. The procedure in this case would be to float the film on a solution of the agent before taking it up on the supporting screen. Only the reagent absorbed by the membrane and the organisms would remain in the specimen.

An application of the technique that is being found particularly useful in this laboratory is the study of the action of bacteriophage. The host organisms are grown to any desired stage, inoculated with a bacteriophage suspension with only slight, if any, disturbance, and then examined at any subsequent time. Since all the organic products of lysis are retained, such micrographs are very nformative (figure 8).

ACKNOWLEDGMENT

SUMMARY

Escherichia coli, Bacillus mycoides, and *Bacillus megathaerium* have been grown successfully on the exposed surface of a thin collodion membrane deposited on a nutrient agar surface. *B. megathcrium* has been grown on the exposed surface of a membrane floating on a synthetic liquid medium. Such preparations can be transferred to the electron microscope specimen screen without disturbing the cells in any way. Electron micrographs of specimens prepared in this way show little, if any, evidence of artifacts.

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THE INTERDEPENDENCE OF MEDIUM CONSTITUENTS IN CITRIC ACID PRODUCTION BY SUBMERGED FERMENTATION[1]

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Extensive work has been done in establishing the optimum conditions for citric acid production by fermentation with *Aspergillus* species (Loesecke, 1945; Karow and Waksman, 1947; Perlman, 1943; Perquin, 1938), and on the use of crude carbohydrate, such as molasses, as raw material (Karow and Waksman, 1947; Gerhardt, Dorrell, and Baldwin, 1947; Perlman, Kita, and Peterson, 1946; Walter, 1947). There are still a number of differences in opinion concerning the essential factors affecting the production of citric acid.

The medium used in the present investigation was prepared with commercial glucose, purified by an aluminum hydroxide coprecipitation method. Systematic variation of the concentrations of the inorganic constituents of this medium gave results indicative of the nature of the relations between certain of the constituents and the accumulation of citric acid in the culture.

EXPERIMENTAL DATA

All experiments were run in shake flasks. The incubation temperature was 25 C. Experimental conditions and methods of analysis were the same as described in previous papers (Shu and Johnson, 1947, 1948). *Aspergillus niger* 72-4 (Shu and Johnson, 1947) was employed in all experiments unless otherwise stated. The composition of the basal medium is given in table 1.

Purification of commercial glucose. In a preliminary experiment it was found that the fermentation of a basal medium prepared from untreated commercial glucose ("cerelose") gave little citric acid (11 per cent of available sugar); however, optimum growth was obtained in this fermentation (1.3 g per 100 ml of medium as compared with 1.7 g per 100 ml in the sucrose control medium). With the addition of iron ions at a level of 1.3 mg per liter of basal medium, untreated commercial glucose gave abundant growth (2.3 g per 100 ml) but produced little acid. In this case the poor yield was probably due to the inorganic impurities present in the commercial glucose. Addition of most of the known growth factors to the fermentation medium has been shown to have little effect on the yield of citric acid (Perlman, 1943). Further, the presence of certain trace metals in the medium has been found to be unfavorable to the production of citric acid (Shu and Johnson, 1947; Perlman, Dorrell, and Johnson, 1946; Bortels, 1927). It seemed advisable to adjust the trace metal content of the

medium by the removal of impurities, followed by the addition of the essential elements in the proper proportions.

An aluminum hydroxide coprecipitation method was employed for glucose purification. To a 500-ml solution containing 140 g glucose (reducing sugar), 1.25 g of $Al_2(SO_4)_3 \cdot 18H_2O$ were added. The pH of the solution was adjusted to 9 with dilute ammonium hydroxide and the resulting precipitate filtered off. To the filtrate another 1.25-g portion of $Al_2(SO_4)_3 \cdot 18H_2O$ was added, the pH adjusted to 7, and the precipitate again filtered off. Analysis of the ash of the filtrate showed less than 50 μg iron, 10 μg copper, 10 μg zinc, and 1 μg manganese ions per 140 g glucose. A basal medium was prepared from this purified glucose solution. Sufficient NH_4NO_3 was added to bring the total N content to 0.87 g per liter. Little growth and poor acid yield were obtained by fermenting such a medium even with the addition of 0.3 mg iron per liter.

TABLE 1

Composition of basal medium

CONSTITUENTS	G PER LITER
Glucose.. .	140
KH_2PO_4 ..	2.5
Nitrogen. .	0.87*
$MgSO_4 \cdot 7H_2O$ ..	0.25
HCl.....	To pH 3.8

* Present as NH_4NO_3 and $(NH_4)_2SO_4$. See text.

The minimum amount of $Al_2(SO_4)_3 \cdot 18H_2O$ necessary in the purification method may be conveniently estimated by a fermentation test. The test involves the determination of growth and titratable acidity after 7 days' fermentation with *A. niger* on the basal medium prepared from glucose treated with various levels of $Al_2(SO_4)_3 \cdot 18H_2O$. A typical example is shown in figure 1. The aluminum sulfate level at which only poor growth and low titratable acid are obtainable (1.8 g in figure 1) may be considered as the quantity of $Al_2(SO_4)_3 \cdot 18H_2O$ needed for sufficient purification.

Essential nature of zinc. It is well known that zinc and iron ions are stimulatory to the growth of *A. niger* (Steinberg, 1919). Iron was found to be necessary for maximum citric acid production, its optimum level varying with the strain of the organism (Perlman, Dorrell, and Johnson, 1946). Regarding the requirement of zinc ion for citric acid production in surface culture, there are many contradictory papers. Perlman, Dorrell, and Johnson (1946) found that the addition of zinc to a highly purified sucrose medium caused a reduction in citric acid yield; Chrzaszcz and Peyros (1935) found no stimulation of acid production when zinc ion was added to the fermentation medium, and Currie (1917) believed that zinc was not essential. On the other hand, Bernhauer (1926), Molliard (1929), Butkewitsch (1923), Porges (1932), Perquin (1938), and But-

kewitsch and Timofeewa (1935) considered that the addition of zinc salts to the fermentation medium was stimulatory to citric acid production.

Zinc was found to be essential for citric acid production in the present investigation. Data on the effect of zinc concentration are presented elsewhere in this
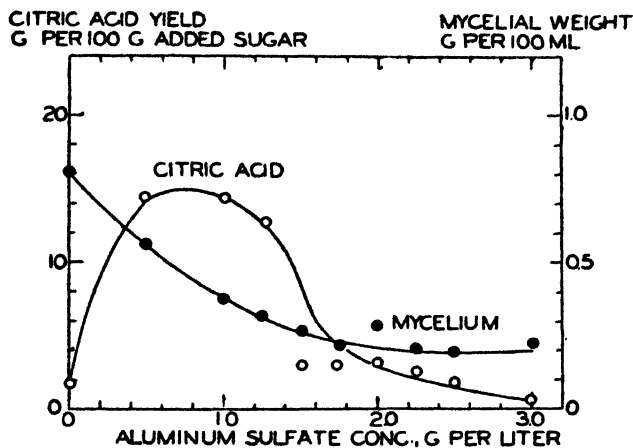
Figure 1. Minimum concentration of aluminum sulfate for cerelose purification. Added aluminum sulfate is expressed as grams of $Al_2(SO_4)_3 \cdot 18H_2O$ per liter of 14 per cent cerelose. Yields are for 7 days' fermentation.

TABLE 2

Zinc requirements of various Aspergillus niger cultures

CULTURE NUMBER	CITRIC ACID YIELD ON ADDED SUGAR		MYCELIAL WEIGHT PER 100 ML	
	With added* zinc ions	Without added zinc ions	With added zinc ions	Without added zinc ions
	per cent	*per cent*	*g*	*g*
59	Trace	Trace	0.3	0.1
62	18†	6†	1.0	0.3
67	47	9	1.2	0.4
68	49	Trace	1.5	0.2
69	18†	9†	0.8	0.3
70	36	9	1.5	0.6
71	40	12†	1.2	0.4
72	51	9†	1.3	0.3
72-4	52	4†	1.2	0.3
73	26	4†	1.2	0.4

* Zinc ions, 0.18 mg per liter, were added to the medium.

† Titratable acid calculated as anhydrous citric acid.

paper. In order to determine whether this requirement for zinc was shared by other citric-acid-producing *A. niger* cultures, 10 strains, obtained from the collection of the Department of Agricultural Bacteriology, were tested for citric acid production, in submerged culture, with and without zinc ions. The results, summarized in table 2, support the conclusion that under the conditions of the

experiment, zinc is required for both growth and acid production by all of the cultures.

Citric acid is not a normal end product of glucose oxidation by A. niger. There are strong indications that only in deficient media does citric acid accumulate. An obvious hypothesis is that citric acid accumulates only when certain components of the mold's respiratory enzyme system are present or absent in insufficient amounts. Thus, a medium deficient in the inorganic constituents required for the synthesis or functioning of certain enzymes involved in the normal metabolic pathway (or enzymes involved in citric acid oxidation) might stimulate the accumulation of citric acid.

Since earlier work (Shu and Johnson, 1948) had shown that the concentrations of some of the constituents of the medium were critical for citric acid production, a systematic investigation of all known essential constituents seemed desirable. After preliminary experiments to establish the proper levels of various constit-

TABLE 3

Composition of the complete medium

CONSTITUENT	G PER LITER
Glucose	140
KH₂PO₄..	2.5
Nitrogen....	1.05*
MgSO₄·7H₂O	0.5
Fe⁺⁺⁺.	0.01
Zn⁺⁺	0.0025
HCl	To pH 3.8

* Present as NH₄NO₃ and (NH₄)₂SO₄. See text.

uents, the medium of table 3 was adopted as containing all essential components in excess. This medium gave good growth but very poor citric acid production.

The concentrations of each of the constituents of this medium were varied in turn, the concentrations of the invariant constituents being held at the levels of table 3. All fermentations were incubated 9 days. When ammonium nitrate was varied, KOH was substituted for NH₄OH in the glucose purification procedure. When KH₂PO₄ was varied, KCl was added to hold the potassium content of the medium constant. In the experiment in which iron was varied, zinc was held at 1.2 mg per liter. The results obtained are summarized in figure 2. It will be seen that two of the constituents, ammonium nitrate and magnesium sulfate (figure 2A, B) had no specific effect on citric acid production. Increasing concentrations of these components caused only a gradual increase in mycelial weight and in the rate of sugar utilization. No large accumulation of citric acid occurred. In the case of the other three components, zinc (C), iron (D), and phosphate (E), another type of behavior was observed. At concentrations of the component below those necessary for optimal growth, citric acid accumulation was observed. At still lower concentrations, growth and sugar utilization were

too poor to make citric acid accumulation possible. It is apparent that when any one of these components is present in amounts limiting growth, citric acid
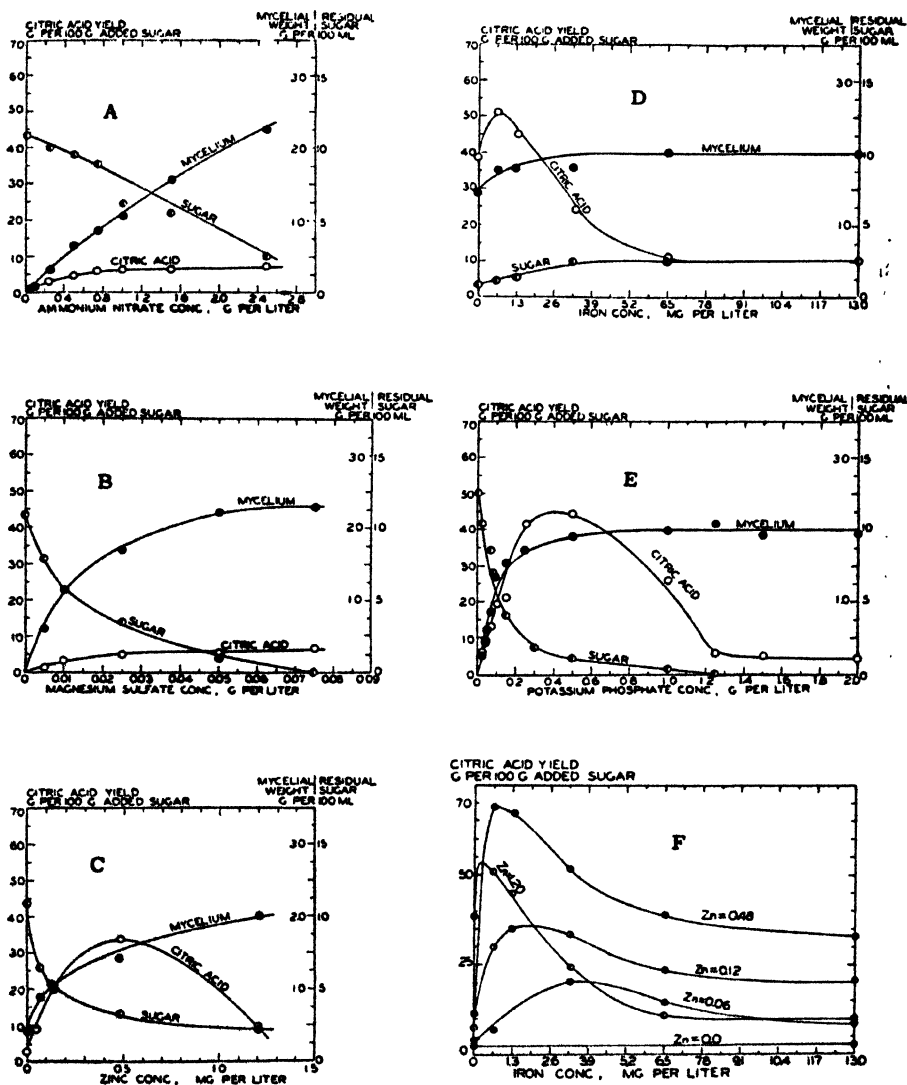
Figure 2. Effect of concentration of essential nutrients.

accumulates, perhaps because of the disturbed functioning of the normal respiratory system.

The yields of citric acid obtainable increase if two of these components (iron and zinc) are both present in limiting concentrations. As may be seen (figure 2F), however, the amount of iron giving optimal citric acid yields depends on the

zinc content of the medium. The experiment of figure 2F, was performed by supplementing the medium of table 1 with various amounts of iron and zinc.

Fermentations with preformed mycelium. Perquin (1938), Szücs (1944), and Karow and Waksman (1947) considered a low phosphate concentration to be essential for maximum citric acid production in submerged culture. These workers all used preformed mycelium in their fermentations. Shu and Johnson (1948), using a combined fermentation and growth medium, found that high phosphate concentrations (up to 5 g per liter of KH_2PO_4) favored high yields. A possible explanation for the discrepancy might lie in the metal content of the media used. Since Shu and Johnson used a medium in which iron and zinc were limiting, high phosphate concentrations were conducive to high yields. Since previous workers did not use purified constituents and did not determine the total amounts of iron and zinc in their media, it appears possible that neither iron, zinc, nitrogen, nor magnesium sulfate was present in limiting concentration in their media. Their fermentations would then be similar to those of figure 2E, in which these constituents were present in excess, and in which good yields could be obtained only in the presence of low phosphate concentrations.

In order to investigate this point, experiments were carried out in which preformed mycelium was used. As in the experiments of the workers referred to above, oxygen rather than air was used as the gas phase in the fermentation flasks. The experimental procedure was the following: The mold mycelium was prepared on 800 ml of Szücs (1944) growth medium containing 0.24 mg zinc and 1.0 mg iron ions per liter. The growth of the mold was carried out on a rotary shaker (320 rpm), in the same way as for the regular fermentation (Shu and Johnson, 1948). After 3 days' growth, the mycelium was collected and washed 5 times with 200-ml portions of sterile distilled water. The washed mycelium was then resuspended in 800 ml Szücs (1944) fermentation medium prepared from purified cerelose. The suspension thus prepared contained about 0.2 g of mycelium per 100 ml. In 50-ml portions this suspension was transferred to previously sterilized 500-ml Erlenmeyer flasks containing various amounts of KH_2PO_4, zinc, and iron. The fermentations were carried out in an oxygen atmosphere, on a reciprocating shaker. The shaker was run at 94 strokes per minute with a stroke length of 4 inches. The oxygen atmosphere was obtained by passing oxygen (1.5 liters per hour), sterilized by passage through a cotton filter, through the flasks, connected in series.

After 7 days' fermentation, citric acid and mycelium weight were determined on duplicate flasks. The experimental results summarized in table 4 indicate that the presence of the phosphate in the medium was desirable for citric acid production, and that simultaneous presence of the phosphate and zinc ions in the medium was even more advantageous.

As may be seen from the data in table 5, when the preformed mycelium was washed only once with 200 ml distilled water, a considerable amount of the acid (50 per cent) was obtained without the addition of phosphate to the medium. Furthermore, in the presence of high concentrations of iron ions (10 mg per liter) or iron (10 mg per liter) and zinc (2.4 mg per liter) ions together, the low

phosphate medium yielded a larger amount of citric acid than the phosphate-rich medium. The maximum citric acid yield (66 per cent) was again obtained from the medium containing high concentrations of phosphate and zinc ions. The data obtained thus indicate that low phosphate concentrations are essential for good yields only when iron and zinc are present in excess. In these experiments, as in the experiments of figure 2, optimal yields were obtained when iron, rather than phosphate, was made limiting.

TABLE 4

Citric acid yield in washed mycelium fermentation

REPLACEMENT MEDIUM PLUS			CITRIC ACID YIELD ON ADDED SUGAR	INCREASE OF MYCELIAL WEIGHT IN 7 DAYS' FERMENTATION*
KH₂PO₄	Zinc ions	Iron ions		
g/L	mg/L	mg/L	per cent	g/100 ml
0	0	0	29	0.5
0	0	10	27	1.1
0	2.4	0	30	0.8
2.5	2.4	0	60	1.6
2.5	0	0	40	1.2

* The initial mycelium weight was 0.2 g per liter medium.

TABLE 5

Citric acid yield with poorly washed mycelium

REPLACEMENT MEDIUM PLUS			CITRIC ACID YIELD ON ADDED SUGAR	INCREASE OF MYCELIAL WEIGHT IN 7 DAYS' FERMENTATION*
KH₂PO₄	Zinc ions	Iron ions		
g/L	mg/L	mg/L	per cent	g/100 ml
0	0	0	50	1.2
2.5	0	0	38	1.4
0	2.4	0	50	1.2
2.5	2.4	0	66	1.8
0	0	10	48	1.2
2.5	0	10	32	1.6
0	2.4	10	33	1.2
2.5	2.4	10	11†	2.2

* The initial mycelium weight was 0.2 g per liter of medium.
† Titratable acid calculated as anhydrous citric acid.

It was thought possible that in a low phosphate medium, manganese, like iron and zinc, might be less inhibitory to citric acid formation than in the regular medium. The fermentations, in replacement medium, were conducted in the manner described above except that the growth medium used in this experiment was the regular high phosphate fermentation medium, and the replacement medium contained 0.24 mg zinc and 1.0 mg iron per liter. Various levels of manganese ions were added aseptically to flasks containing replacement medium in which 3-day-old preformed mycelium was suspended. The fermentation was

done in an oxygen atmosphere on the reciprocating shaker. In another series of flasks, regular fermentations were made in high phosphate medium on the rotary shaker. After 3 days' shaking, various levels of manganese ion were added aseptically to these flasks. These flasks were then similarly shaken on the reciprocating shaker in oxygen atmosphere. After 7 days' incubation, titratable acid was determined on both series of flasks. The results are shown in figure 3. It may be seen that the inhibitory effect of manganese ion was less pronounced for the fermentation in low phosphate replacement medium than for that in high phosphate medium. For example, at a manganese level of 6 μg per liter the yield was increased 3-fold in the absence of phosphate.
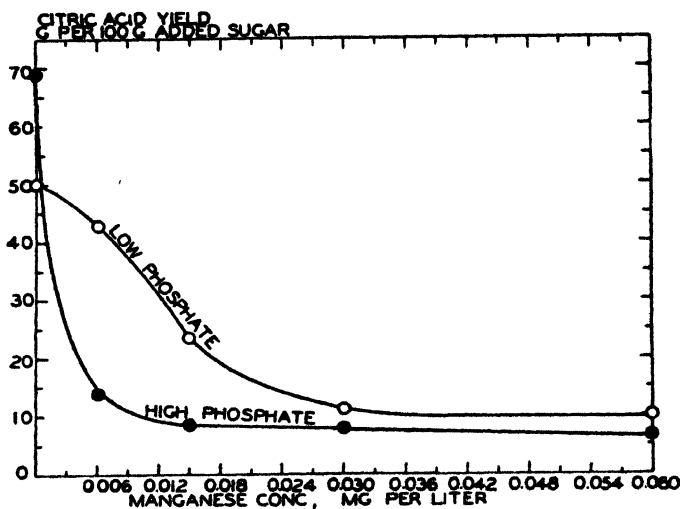
Figure 3. Effect of the presence of manganese ions in high and low phosphate media.

SUMMARY

An aluminum hydroxide coprecipitation method was shown to be satisfactory in the purification of commercial glucose for submerged citric acid fermentation. After treatment by this method and the addition of 0.3 mg zinc and 1.3 mg iron ions per liter of medium, commercial glucose gave a citric acid yield of 67 g per 100 g of added glucose on a 14 per cent sugar solution. The fermentation required 9 days.

Among the 5 essential constituents of the fermentation medium (NH_4NO_3, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, zinc, and iron), KH_2PO_4, zinc, and iron affected the yield of citric acid by some means other than their effect on growth. Their optimum concentrations for maximum acid production are interdependent.

In the fermentation of a phosphate-free replacement medium with preformed mycelium, the addition of KH_2PO_4 and zinc favored the production of citric acid. However, the reduction of citric acid yield by the presence of manganese ions, and by high concentrations of iron and zinc ions, was less pronounced in a phosphate-free medium.

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AN UNUSUAL STRAIN OF STREPTOCOCCUS ISOLATED FROM SUBACUTE BACTERIAL ENDOCARDITIS

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Streptococci isolated from man and animals are usually nonmotile. Only sixteen strains of motile streptococci have been reported (Koblmüller, 1935; Pownall, 1935). All of these strains belonged to the enterococcus group. Enterococci are frequently isolated from the blood stream. The occurrence of motile strains, from man or animals, has not as yet been reported in America.

The taxonomy of enterococci was greatly clarified by Sherman (1938), Kuehner (1946), and in the recent edition of *Bergey's Manual* (Breed *et al.*, 1948). The cardinal points in the differentiation of enterococci from other streptococci are hemolytic ability and tolerance for the following: heat (60 C for 30 minutes), methylene blue (0.1 per cent solution), sodium chloride (6.5 per cent), bile, and alkaline medium (9.6 pH). In addition, the action upon milk, esculin, sodium hippurate, gelatin, starch, and a number of other carbohydrates (chiefly lactose, inulin, raffinose, mannitol, sorbitol, and trehalose) aids in the classification of these orgranisms.

The motile streptococcus studied by us was isolated from the blood stream of an adult woman suffering from subacute bacterial endocarditis.

The cultures of the organism consisted of ovoid cells, 0.75 to 1.0 μ in diameter, occurring in pairs and short chains. It was gram-positive and encapsulated, motile with one flagellum at room and incubator (37 C) temperature. The flagellum was difficult to stain. After futile experiments to make it visible by Leifson's method, nigrosin permitted us to see it. The difficulties encountered in the staining of flagella of motile streptococci were described by Pownall (1935).

The organism grew well at room temperatue and at 37 C. Gelatin was not liquefied. On nutrient agar small, round, raised colonies were formed. The growth in broth was diffuse, later forming a heavy sediment. Transitory acidity in litmus milk, without reduction, and alpha type hemolysis on blood agar plates were observed. Acid but no gas was produced from glucose, sucrose, maltose, mannitol, salicin, xylose, trehalose, and arabinose in 1 day; from lactose in 3 days; from glycerol in 7 days. Raffinose, sorbitol, inulin, rhamnose, and dulcitol were not attacked in 3 weeks. No dextran was formed. Esculin was split. Starch and sodium hippurate were hydrolyzed. Ammonia was formed from peptone. The organism survived heating to 60 C for 20 but not 30 minutes. It grew on agar at pH 9.6; in the presence of 50 per cent bile; in the presence of 0.1 per cent methylene blue; and on 6.5 per cent sodium chloride agar. The fibrinolysin test was negative. It was sensitive to 0.6 units of penicillin and to 25 μg of streptomycin.

This microbe differed from the *Streptococcus pyogenes* group by the fermentation of mannitol, the tolerance of methylene blue, and hemolytic properties. It was distinguished from the viridans group of streptococci by its tolerance of methylene blue, alkalinity, and salt and also by the production of ammonia. It could not be placed as a typical member of the "lactic" or the "enterococcus" group because of its lack of action on milk.

Thus this streptococcus cannot be classified as a typical member of any of the commonly recognized groups. It may, however, be a variant of *Streptococcus faecalis*, which belongs to the enterococcus group. It differs from this organism only by its failure to attack milk and by its ability to hydrolyze starch.

SUMMARY

An unusual motile streptococcus isolated from the blood stream of a patient suffering from subacute bacterial endocarditis is described.

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ELECTRON MICROGRAPH STUDIES OF TWO STRAINS OF PLEUROPNEUMONIALIKE (L) ORGANISMS OF HUMAN DERIVATION

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The organisms of the pleuropneumonia group have been set apart from other microorganisms because of their small size and curious morphology. Their extraordinary pleomorphism is well illustrated in the studies that Tang made by means of dark-field observation of living broth cultures (Tang *et al.*, 1934, 1936). He described granules, coccobacillary bodies, filaments, and "ring" forms; and he recognized that the latter were not actual rings, as some authors have supposed, but were solid bodies of the sort that have come to be called "large round bodies" or simply "large bodies." Tang noted that the large bodies were formed from the granules and came to contain one or more dots that increased in size to form new granules, which then budded off from the large bodies. In freshly isolated strains the various elements remained attached to one another in filamentous forms, and these filaments broke up into round or rod-shaped forms. When several granules remained attached by short filaments to different points on the surface of a single large body, the resultant structure had a starlike shape, which led to the name "*Asterococcus*," sometimes applied to these organisms.

Similar observations have been reported by other authors, as reviewed by Dienes (1945). Tang's studies were made with strains of *Pleuropneumonia boris*. The importation of this organism into the Untied States is prohibited by law because of its virulence for cattle, but organisms of similar morphology have been isolated in this country and elsewhere from other animals. Some of these strains appeared as saprophytes upon mucous membranes, others as agents of spontaneous joint infections in rats. In human beings organisms of this sort are common inhabitants of the uterine cervix. They have also been isolated from various genitourinary infections, notably prostatitis. In a series of 23 men with prostatitis yielding these organisms, acute joint involvement occurred in 11 cases at the time the prostatic cultures were positive. The organisms were cultivated directly from joint effusions in two of these patients (Dienes *et al.*, 1948).

The strains from these diverse sources, differing in pathogenicity and in serological properties, have in common the fact that they are among the smallest organisms that have been grown on lifeless media, and that they resemble each other in their unusual morphology and distinctive, almost microscopic, colonies. For these reasons they have been classed together as organisms of a "pleuro-

pneumonia group," or "pleuropneumonialike organisms," or, as a brief designation, "L organisms" (Sabin, 1941).

Although morphology, including size as well as shape, is the basis of this classification, much debate has centered over the actual structure and nature of these minute, pleomorphic organisms (Klieneberger and Smiles, 1942). We felt that the greater resolution made possible by the electron microscope might be of some aid in this problem. The present paper contains electron micrographs of strains of human origin supplied by Dr. Louis Dienes. These will be referred to as L organisms.

METHODS

All electron micrographs were made at 55 kv.

L strains were grown on ascitic fluid peptic digest plates at 37 C and examined with the light microscope by means of wet cutout preparations stained with methylene blue and "azur II." This technique has been described elsewhere, together with notes on the preparation of the medium (Dienes et al., 1948). The peptic digest was a modification of "Martin's peptone" (Wadsworth, 1947). It was prepared by adding 600 g of fresh, minced hog stomach and 36 ml conc. HCl to 3,000 ml water. After 20 hours' incubation at 50 C, the fluid was decanted and buffered with 4 g K_2HPO_4 and brought to pH 7.8 with 10 N NaOH. It was then filtered through gauze and cleared by filtration through paper. Sterilization was accomplished by passage through a Seitz filter. When plates were to be prepared, 15 per cent of this digest and 25 per cent of ascitic fluid were added to the sedimented boiled blood agar previously described. The digest affords more abundant growth, but its use is not essential. It is important that the ascitic fluid be buffered to pH 7.4 with KH_2PO_4 and that the plates be soft (1 per cent agar) and sealed to prevent drying during storage. L colonies grow down into the agar and hard plates can completely prevent growth.

Although the plates just described permitted excellent growth and serial passage, neopeptone infusion broth enriched with ascitic fluid and peptic digest failed to support continued propagation of two L strains which we isolated from the human uterine cervix. Rabbit serum proved equally unsatisfactory as an enriching material, but when 30 per cent horse serum was added to the broth the strains were successfully carried through 8 transfers. The tubes developed only faint turbidity, but subcultures to plates yielded abundant growth.

Preparations for electron micrography were made by touching a few colonies with a pin, which was then repeatedly thrust into a drop of distilled water upon a parlodion film covering the electron microscope screen. When it was desired to study fixed organisms, a drop of 2 per cent osmic acid solution was put upon the screen instead of a drop of water. This fixative volatilized without depositing crystals. A second method was to flush the osmic acid solution back and forth with a pipette over the surface of agar bearing colonies. A drop was then transferred to a screen. The latter method was much less satisfactory, since debris was carried over to the screen.

We are indebted to Dr. Keith Porter and Mr. Winfield Baker for assistance with some of the micrographs.

Findings with Organisms of the Pleuropneumonia Group (L Organisms) Isolated from Human Sources

The L50 strain was the first to be examined. It had been isolated from the uterine cervix and carried for several years on ascitic peptic digest plates as a type culture representative of L organisms derived from the genital tract. A plate bearing colonies of this strain was provided by Dr. Dienes. The plate had been incubated 4 days, and a semiconfluent growth of tiny colonies approximately 0.2 mm in diameter had developed upon it. Stained wet cutout preparations, examined with the oil immersion lens, showed that these colonies were composed of round bodies of varying size and small rod-shaped bodies, and that they had the peculiarly distinctive appearance of L colonies, photographs of which have been published elsewhere (Dienes, 1945).

Electron micrographs of organisms of this strain are given in figures 1 to 5. They show round bodies that vary in size from 0.5 (figures 2 and 3) to 3 microns (figure 5) in diameter. In addition, short curved or twisted and slightly nodular filaments were seen, the longest measuring 5 microns. Of particular interest was the observation of rod-shaped bodies. Many such were found. They averaged 1.3 microns in length and 0.5 microns in width. They thus had a distinctly elongated, bacillary shape. In figure 1 the protoplasm of one of these rod-shaped bodies has retracted slightly, and a well-defined cell wall is visible. The size and shape of these organisms, together with the demonstration of a cell wall, make it certain that they must be regarded as indubitable bacterial cells. The smallest cells seen in this strain were the 0.5-micron round bodies mentioned above.

The cytoplasm of most of the cells in figures 1 and 2 appeared vacuolated. In addition, in these pictures and in figure 3, well-defined intracellular granules 0.1 to 0.18 microns in diameter were seen. These granules occurred independently of vacuolation of the cells. The largest cell in figure 1 shows them distributed rather evenly along the course of the cell. Larger and less well-defined areas of increased density can be seen in the elongated cell shown in figure 3. A large, sharply defined area of increased density, 0.3 microns across, occurred at the enlarged end of the cell in figure 4. Short, twisted, nodular filaments or chains of small round cells are characteristic of the L type of growth. The long cell in figure 3 would appear to be such a filament. The large, rounded, dense knob at the end of the cell in figure 4 probably represents an early stage in the formation of a free round cell.

Of the three round cells shown in the micrographs, the smallest (figure 2), at the voltage employed, appeared as a homogeneous protoplast surrounded by a peripheral structure of relatively low density. The second, slightly larger (figure 3), contained five scattered areas of increased density. The large round body of figure 5 had a large diffuse area of increased density in its center. New round or elongated cells are known to arise from large bodies such as this. The body shown here was selected because its two stubby outpouchings seemed to represent the earliest stage in such germination. More will be said of the process of germination in this and the succeeding paper.

Round bodies were considerably less common in the electron micrographs than
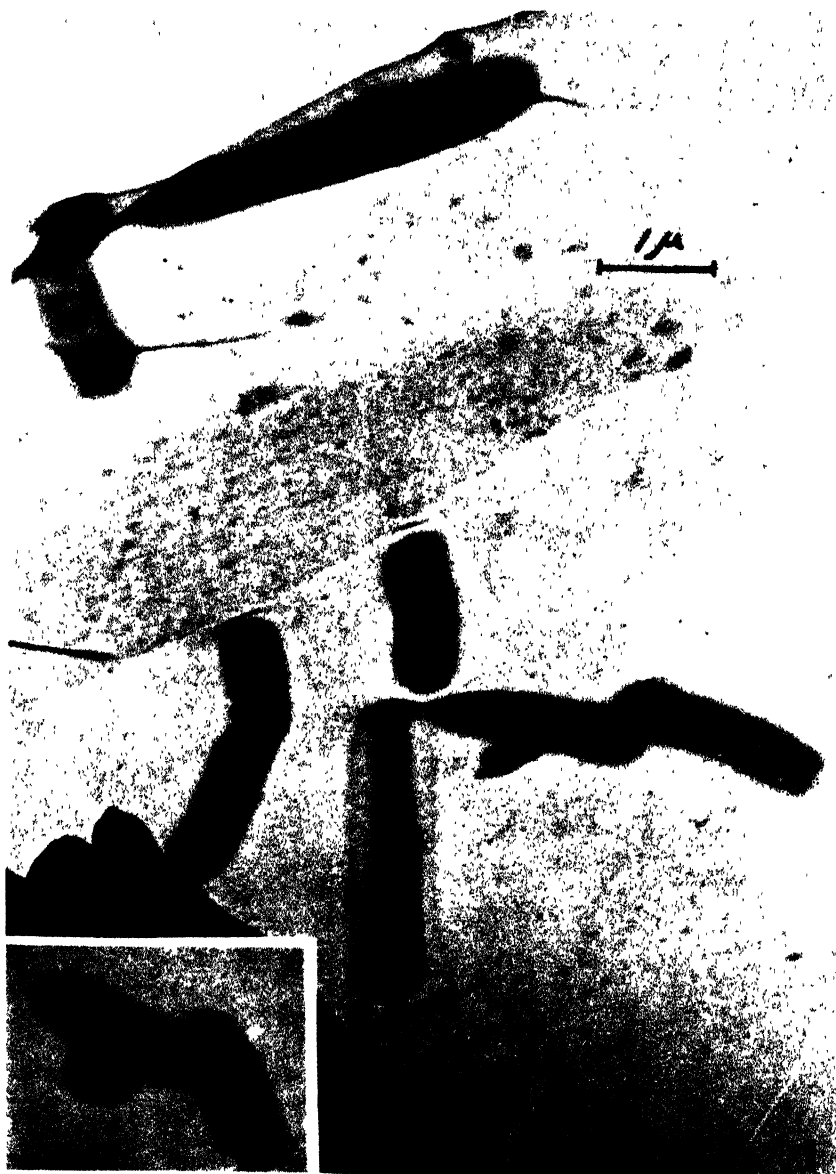
Figures 1 to 17 were made with an electron microscope at 55 kv.
Figure 1. L50. Rod-shaped cells containing intracellular granules. In the big cell at the lower center of the photograph the protoplasm has retracted and the cell wall is visible. 15,000 ×.
Figure 2. L50. Two rod-shaped cells and a round cell. 15,000 ×.

in wet cutout preparations, and it was suspected that some procedure in the preparation of the screens may have acted to destroy them. Well-developed cell walls were not detected on the round bodies, a finding which suggested that their

surface might be less sturdy than the surface of the bacillary forms.   Hence it seemed possible that the loss of round bodies had resulted from osmotic bursting occasioned by immersion in distilled water when the screens were prepared.
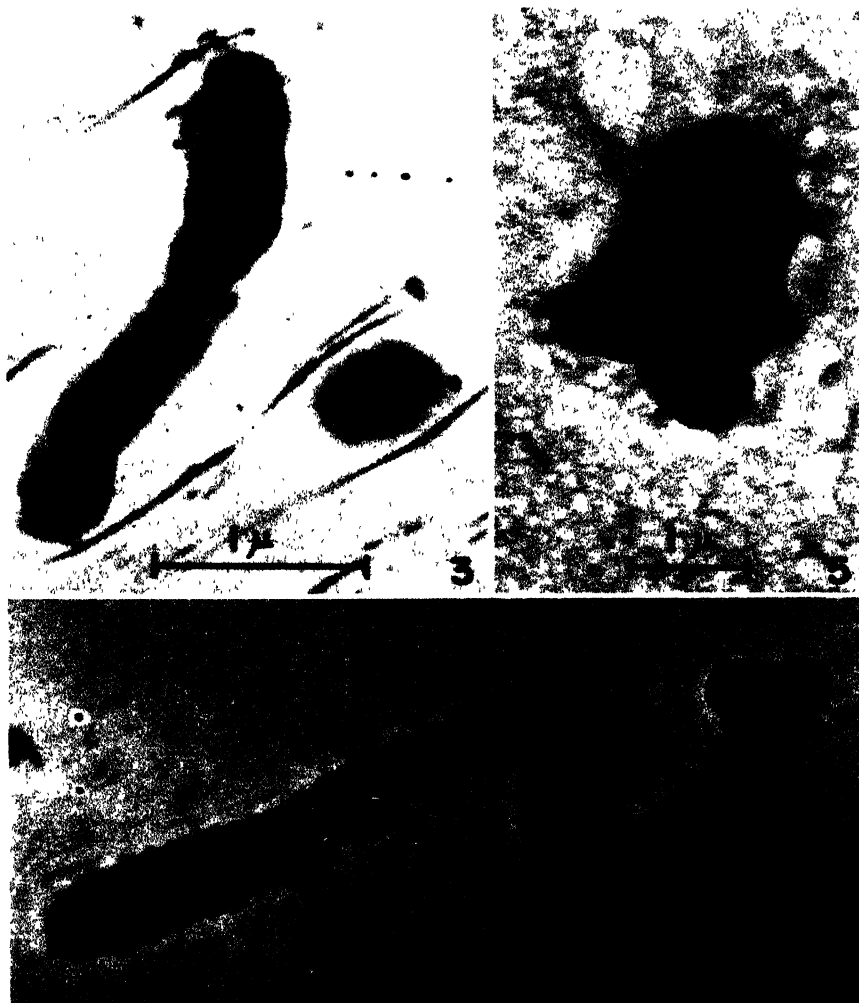
Figure 3. L50.   A nodular, filamentous cell and a round cell.   27,000 ×.
Figure 4. L50.   Filamentous cell with enlargement at one end.   27,000 ×.
Figure 5. L50.   Large round body.   Its uneven contour and the two blunt outgrowths at its lower pole illustrate the earliest stage of germination.   15,000 ×.

This hypothesis was borne out by subsequent observations reported in this and the accompanying paper.

The next strain examined, L4330, had been isolated from the uterine cervix in 1940.   Except for intermittent periods of preservation in the frozen state, it had been carried since then on ascitic peptic digest plates by transfers every 4

days in Dr. Dienes' laboratory.   In 1942 this strain was sent to Dr. Albert Sabin, who published an account of studies made with it in his laboratory, concluding that it should properly be classified in the pleuropneumonia group (Warren and Sabin, 1942).   When sent to us in 1945, it formed colonies and exhibited pleomorphic forms like those shown in the photographs published by Warren and Sabin in connection with their study of it.   In wet cutout preparations it appeared comparable to other L strains examined by this method.

Because of the small size of the colonies and their habit of burrowing down into the agar rather than heaping up as surface growths, transfers of L strains from plate to plate are customarily made by cutting out a piece of agar and rubbing it over the surface of the plate to be inoculated.   L4330, transferred in this way, produced densely crowded growths that appeared as thin, delicately stippled films on the agar after 4 days' incubation.   With the oil immersion lens, such a film was seen to be made up of tiny, discrete colonies, buried in the agar and composed of minute round forms that stained a deep blue in the cutout preparations.   Pleomorphic forms were not in evidence.

Very different were the findings in less densely inoculated areas.   Ascitic peptic digest plates were inoculated with L4330 by rubbing a small piece of agar bearing colonies over one-third of its surface (area 1), then cutting out a block from the inoculated area and rubbing it over another third of the plate (area 2), and finally inoculating the last third (area 3) in the same way with a piece from area 2.   After 4 days' incubation, the growth in area 1 seemed confluent.   In area 2 the colonies were almost everywhere discrete, separated by about 1 mm from each other.   These colonies were about 0.2 mm across.   In area 3 the colonies were well separated, 2 to 3 mm apart, and were about 0.5 mm in size.

Cutout preparations examined with the oil immersion lens showed that the colonies in the seemingly confluent part of area 1 were discrete, though almost touching each other.   These colonies averaged 10 to 20 microns in diameter. Each was a rather compact mass of tiny, deeply stained granules growing down into the agar.   None of the granules appeared to be larger than 0.5 microns. Large round bodies, bacillary forms, or filaments could not be found, but some granules were connected by a fine thread.

In area 2, where the colonies were less crowded and bigger, their appearance was different.   They still showed the deeply stained granules growing down in the agar, but each had a mound of surface growth that appeared as a lacy halo when observed with the low power of the microscope.   With the oil immersion lens, this halo was seen to be composed of large round bodies 5 to 7 microns in diameter, as shown in many published photographs of L colonies.   The colonies in area 3 were similar but larger, and in them new growths of granules down into the agar had begun at many places on the periphery of the halos.   In these areas some of the "granules" were distinctly rod-shaped instead of round.

This experiment is cited in detail because bacillary forms were seldom seen in electron micrographs made from confluent areas of growth.   To observe them it was necessary to employ screens prepared from well-separated colonies.   They were sought in particular with the aim of elucidating the relationship between the rod-shaped and the round bodies.

It was soon learned that the round bodies in this strain, as in L50, were in large measure destroyed by immersion in distilled water. Figure 6 shows organisms plasmolysed as a result of such treatment. These plasmolyzed cells show clearly the reason for their destruction, for it is evident that they lack the sturdy outer structure that composes the wall of ordinary bacteria. One to five small dense granules lay along the limiting surface, presenting exactly the picture of "ring" forms long known to be characteristic of pleuropneumonia organisms. One such granule lay in each end of a rod-shaped cell residue (upper right of photograph), and two lay in the enlarged end of a longer rod-shaped cell residue with two smaller and fainter ones in the narrower portion of the same cell (lower left). The blurred appearance of the rod-shaped cells showed that they also suffered osmotic injury as did the round cells. The delicacy of their cell wall is apparent. The presence of granules in injured cells is obviously difficult to interpret. They are commented upon merely because they conform to the pattern observed in dark-field or stained preparations.

Because of the plasmolysis, it was determined to employ a fixative. For this purpose, a drop of 2 per cent solution of osmic acid was placed on the screen instead of a drop of distilled water, and the organisms were transferred to it by a pin. This procedure preserved them. Electron micrographs of L4330 made in this manner are given in figures 7 to 17.

The round forms observed in the fixed specimens ranged in diameter from 0.25 to 0.7 microns. They had a finely granular appearance, occasionally with a dense mass in the center (figure 7). These small round cells were found to be capable of division by simple fission in the manner of cocci (figures 8 and 9). Some of the small round cells became elongated, and it appeared that the rod-shaped cells arose in this way (figures 7 and 10). Rod-shaped cells from 0.5 to 1.3 microns in length were found (figures 11 to 15). The larger of these exhibited a swelling toward one end.

The formation of a new round cell ("large body") from this swelling is well shown in figures 11 and 15, which also show concentration of material with greater ability to scatter the electron beam in the forming large body, a picture reminiscent of that obtained with developing spores.

The germination of large bodies to form a new generation of small, round cells is shown in figures 16 and 17. Figure 16 shows a large body whose surface has become nodular, resulting in a mulberrylike appearance. Observation of living cultures has shown that such bodies break down into multiple new daughter cells without much further change in shape. The large body (A) in figure 17 had given rise to two thick outgrowths, B and C. B was a blunt mass, but C had begun to differentiate into small round cells, three of which are clearly defined. The invagination of the outgrowing filament to form these three cells marks the process as one of segmentation of the filament.

Observations on Nelson's Coccobacilliform Bodies

A further group of organisms of interest in connection with the present work were the "coccobacilliform bodies" isolated from the nasopharynx of rats, mice, and chickens (Nelson, 1939). Not all of these strains have been grown on life-
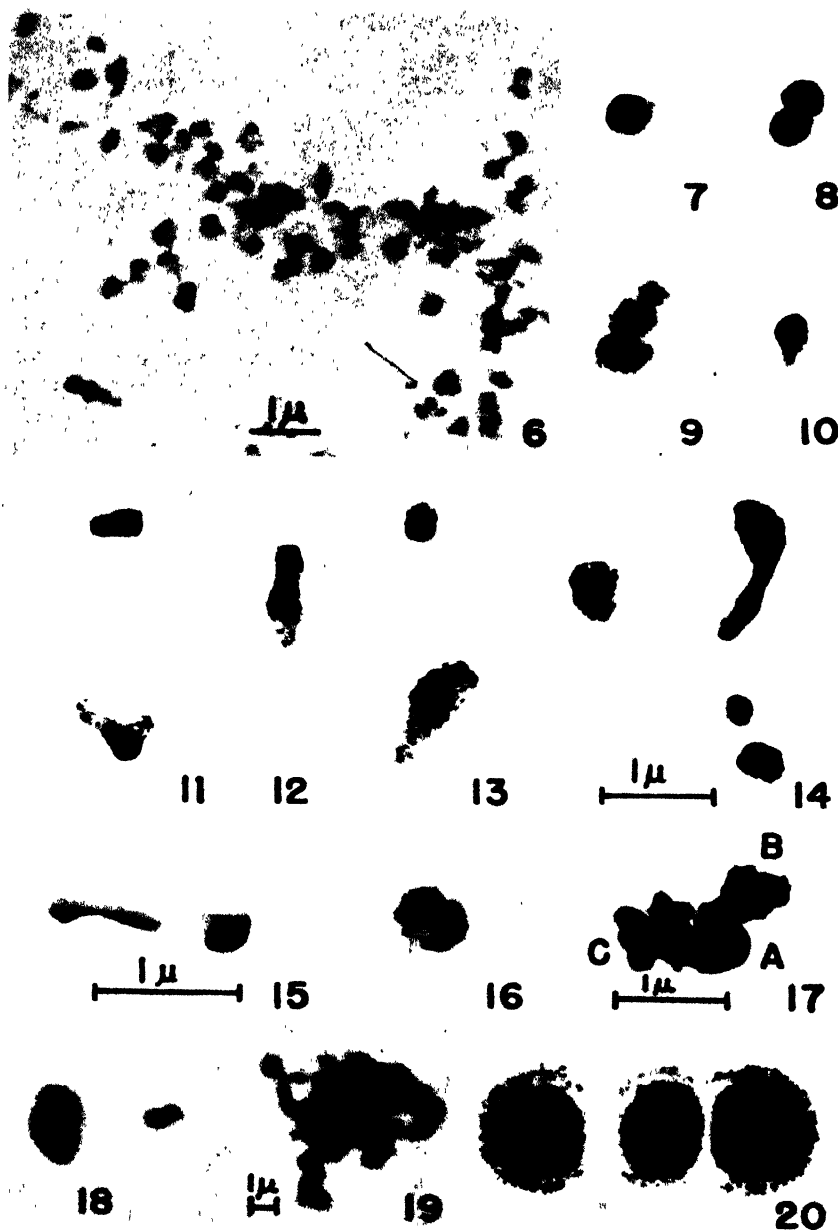
Figure 6. L4330.   Cells plasmolyzed on a screen prepared from distilled water.   Ordinary bacteria are not obviously damaged by similar preparation.   Plasmolysis of the L organisms illustrates an important property of their surface, namely, its fragility.   9,000 ×.
   Figures 7–17. L-4330.   Osmic acid fixation.   All are 15,000 × except figure 15, which is 20,000 ×.
   Figure 7. Round cell with dark granule in center.
   Figure 8. Two round cells dividing by simple fission.

less media, but it is probable that they should be included in the pleuropneumonia group (Sabin, 1941). A strain of these organisms isolated from chickens and adapted to *in vitro* cultivation was provided by Dr. Nelson. This was an avirulent strain. Nelson found that virulent strains failed to grow on media that supported growth of organisms of the pleuropneumonia group. When inoculated onto ascitic peptic digest plates, it produced abundant growths of tiny, glistening pin-point colonies. These proved readily transplantable, but the colonies were always tiny, never attaining a diameter greater than 0.3 mm. Growth was less abundant and the colonies tended to autolyze on horse serum or horse blood plates. In liquid media excellent growth was obtained in infusion broth enriched with 30 per cent horse serum, definite turbidity appearing after 72 hours' incubation and enormous numbers of colonies arising when a drop was transferred to a plate. No growth occurred in plain broth or in broth enriched with ascitic fluid. Good growth took place in Tyrode's solution containing bits of chick embryo tissue and buffered to pH 7.4 with Na_2CO_3. Although growth in this medium did not progress to the extent of producing turbidity, transfers from it to plates yielded great numbers of colonies. Giemsa-stained smears were prepared from the liquid media, and the tiny round coccoid and minute rod-shaped bodies described in the literature were observed.

More definitive observations, for our purposes, were made by an examination of pieces of agar cut out of ascitic peptic digest plates bearing the colonies and stained as wet preparations by methylene blue and "azur II" in the manner employed for L organisms. With this technique the individual cells could be clearly seen lying on or in the agar, not shrunk by drying as in smears, and their relation one to the other could be clearly ascertained. During the first 48 to 72 hours of growth, the colonies consisted of tiny round bodies about 0.5 microns in diameter. These lay singly, in pairs, or in short, twisted chains, bespeaking division by simple fission. The colonies grew down into the agar. Larger round bodies, up to 2.5 microns in diameter, occurred, notably at the surface of the colonies or

Figure 9. One of the "short, nodular filaments" characteristic of L organisms. It is composed of 3 round cells that have divided by fission.
Figure 10. Elongated cell.
Figure 11. Two rod-shaped cells, one of which is enlarged at one end. This illustrates the beginning of large body formation.
Figure 12. Rod-shaped cell.
Figure 13. Rod-shaped cell with beginning enlargement toward one end. Also a small round cell.
Figure 14. Rod-shaped cell with bulbous enlargement at one end. Also 3 round cells.
Figure 15. Filamentous cell with well-formed large round body at one end.
Figure 16. Large round body showing beginning segmentation into multiple small round cells.
Figure 17. Large round body (A) showing outgrowths at B and C. The outgrowth at C has begun to segment into small round cells.
Figures 18-20. Light microscope photographs of a strain of Nelson's coccobacilliform bodies. Agar cutout preparations stained with methylene blue and "azur II."
Figure 18. Large round body on surface of agar. 4,000 X.
Figure 19. Young colony composed of short tortuous chains of small round cells growing down into the agar. The central dark area was composed of a large round body lying on the surface of the agar and consequently somewhat out of focus. The chains of small round cells grew out from the large body. 4,000 X.
Figure 20. Four-day-old colonies on agar. The dense center and lacy peripheral zone are characteristic of organisms of the pleuropneumonia group. 100 X.

singly on the agar (figure 18). Although the colonies consisted for the most part only of the small cells of rather uniform diameter, it was evident that growth sometimes derived from the larger round bodies, one or more chains of small cells extending out from them. Figure 19 shows one such young colony that had developed from a large body. At least three twisted nodular filaments extended out from the body, and each filament had begun to segment into small round cells. These filaments had grown down into the agar, and the large body that lay on the surface was, therefore, slightly out of focus. Next to this young colony, two small round cells lay on the surface of the agar. They were evidently in the process of division by simple fission. The small round cells in this photograph were all approximately 0.5 microns in diameter. Older, better-developed colonies are shown in figure 20. They have the dark center and lacy peripheral halo associated with colonies of the recognized strains of pleuropneumonialike organisms. Each tiny dot in this halo was a single small round or minute rod-shaped cell, with larger round cells here and there, as higher magnification showed.

The appearance of the colonies, their habit of burrowing down into the agar, the size and morphology of the elements composing them, and the reproduction both by simple fission and by multipolar germination of large bodies led us to conclude that this strain of coccobacilliform bodies could properly be included in the group of pleuropneumonialike (L) organisms.

DISCUSSION

By means of the electron microscope it has been possible to observe the pleomorphic cells of two strains of L organisms with greater accuracy than had been possible with light microscopy. The rod-shaped cells of the L50 strain were thus found to have a cell wall that could be distinguished from the inner cell protoplasm. The somewhat larger, more filamentous cells developed nodular swellings, thereby revealing the manner of formation of the large round bodies. The organisms of the L4330 strain were smaller, and differentiated cell walls were not detected in them. Their pattern of development was nevertheless similar, and a series of micrographs is given showing the development of large bodies in this strain. When these bodies began to form in rod-shaped cells, one saw an enlargement in the middle or toward the end of the cell. This enlargement increased in size until it became very bulbous. Such enlarged areas usually caused more scattering of the electron beam than did the other portions of the cells bearing them.

The germination of large bodies to yield a new generation of small round cells was also observed. When this occurred, the protoplasm pushed outward at 2 to 3 points on the surface of the body. These outgrowing filaments then became constricted in several places along their course, and the new cells were produced by fission of the filaments. This type of germination might be compared to the germination of certain *Actinomyces* spores from which four filaments grow out and then segment to yield new cells (Knaysi, 1944). It differs from the

germination of most bacterial spores in that it is multipolar rather than unipolar. Sometimes the whole surface of a large body became nodular, only very stumpy filaments were extended, and segmentation into new cells occurred while the body was still essentially round.

Consideration of the differences between large bodies and bacterial spores appeared to throw some light upon the manner of their germination. The resistance of spores to heat, changes in osmotic environment, drying, and aging is considered to be a property conferred upon them in large part by the character of their cell walls, which have been shown by various techniques, including electron micrography (Knaysi *et al.*, 1947), to be well-developed structures. Upon germination, the wall cracks, and the new vegetative cell then pushes out. On the other hand, our electron micrographs showed that the large bodies did not have the well-formed, sturdy cell walls possessed by spores. The lack of this structure doubtless accounts in part for the fact that they are not resistant to heat, drying, or aging. That their limiting membrane is indeed delicate was shown by the finding that they were readily burst by immersion in distilled water. This quality of the surface of the large bodies would seem to go some way toward explaining the multipolar character of their germination; for, at the time of germination, an increase in the volume of protoplasm contained within such a delicate membrane might be expected to weaken the membrane at many points, through which the protoplasm could push outward. Individual differences in elasticity of the surface may perhaps determine whether a given large body will send out a few relatively long filaments, which then segment, or whether its surface will become nodular and segmentation occur without the development of filamentous outpouchings. The latter process is well described in the case of the breakdown of large bodies of L1 into multiple small round forms (Klieneberger and Smiles, 1942).

It is abundantly clear that reproduction by means of large bodies is not the sole means of growth of the L organisms. Under certain conditions, notably crowding of colonies, one sees no forms other than small round cells, and these are obviously reproducing by simple fission in the manner of cocci. Our studies with the electron microscope show that these small round cells, as well as the rod-shaped cells, are capable of division by simple fission. It would seem likely that large round bodies also developed from the small round cells, for large bodies often formed in colonies in which rod-shaped cells were not in evidence.

Indubitable bacillary forms were demonstrated by Weiss (1944) in electron micrographs of a strain of organisms of the pleuropneumonia group isolated from a spontaneous joint infection in a rat. Multipolar germination of a large round form is well seen in figure 10 of her paper. The frank bacillary forms detected in her cultures resemble those seen in the L50 strain, in which such forms were larger and more numerous than is usually the case in strains isolated from the human genital tract. The smaller cells of the L4330 strain are more typical of freshly isolated human strains.

Recently, Hesselbrock and Foshay (1945) published studies on the morphology

and filterability of *Bacterium tularense* that led them to conclude that that organism should be included in the pleuropneumonia group. Their photographs show well-defined bacillary forms, and also large round bodies from which filaments, sometimes branched, have grown out.

The identification of rod-shaped cells in the two human L strains described in this paper, and their presence in the photographs of Weiss and of Hesselbrock and Foshay, support Dienes' view (1945) that the organisms of the pleuropneumonia group are essentially small, pleomorphic bacilli. The development of large round bodies as swellings within bacilli or bacillary filaments and the manner of subsequent germination of these bodies afford an explanation of the significance and function of the pleomorphism. The main point that distinguishes the germination of the large bodies is that they divide into multiple daughter cells. Formation of such large bodies and reproduction by means of them are not limited, however, to the tiny organisms now lumped together in the pleuropneumonia group. It has been observed in large bacteria belonging to several of the common genera. In the accompanying paper electron micrographs are presented that show this mode of reproduction in a large anaerobic bacterium, *Bacteroides funduliformis* (Smith, Mudd, and Hillier, 1948).

SUMMARY

Two strains of pleuropneumonialike (L) organisms derived from the human genital tract were studied. The morphology and mode of reproduction of these organisms as revealed by the electron microscope are described. One strain of Nelson's coccobacilliform bodies was examined. Its morphology and colonial appearance correspond to those of recognized strains of pleuropneumonialike organisms.

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L-TYPE VARIATION AND BACTERIAL REPRODUCTION BY LARGE BODIES AS SEEN IN ELECTRON MICROGRAPHIC STUDIES OF BACTEROIDES FUNDULIFORMIS

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The division of one cell into two daughter cells has long been accepted as the mode of reproduction of the bacteria. A second mode of reproduction, however, has been observed in many strains, belonging to several common genera, and consists in the formation of large round bodies from which more than two daughter cells derive (Dienes, 1946, 1947). This process is exhibited very commonly by organisms of the pleuropneumonia group (Smith, Hillier, and Mudd, 1948). It is also often seen in the large anaerobic bacteria of the *Bacteroides funduliformis* group, and the production of multiple daughter cells from *Bacteroides* large bodies has been demonstrated by cinematographic records of the germination of individual living large bodies (Dienes and Smith, 1944). The daughter cells resulting from such germination usually resemble the relatively large bacillary *Bacteroides* cells; but under certain conditions much smaller elements are produced, and these form tiny colonies with the peculiarly distinctive appearance characteristic of colonies of the pleuropneumonia (L) group of organisms. The production by ordinary bacteria of such tiny L-type colonies has been termed L-type variation. The formation of large bodies has been a prominent feature in all strains of bacteria that have exhibited L-type variation, no matter what their genus, and the L-type of growth has derived from the large bodies.

The work reported in the present paper was undertaken with the aim of gathering further information about this process by means of the electron microscope. *Bacteroides funduliformis*, strain 132, was selected as the test organism. It had been isolated from a kidney abscess (Smith and Ropes, 1945). The paper just cited contains a review of the clinical aspects of *Bacteroides* infections.

METHODS

The 132 strain was grown in Brewer's thioglycolate broth. Cells for electron microscopy were obtained by diluting the broth with an equal part of Tyrode's solution and centrifuging the organisms down.

The sediment was resuspended in Tyrode's solution, spun again, and resuspended in the solution a second time. The washing freed the cells of material in the broth that otherwise clouded the electron pictures. This suspension was divided into 4 tubes, which were then centrifuged. The sediment in tube 1 was taken up in a small amount of Tyrode's solution and drops were transferred to parlodion films on screens. After drying, these screens were floated on distilled

water to dissolve salt crystals. The sediment in tube 2 was suspended in 0.1 per cent phosphotungstic acid (PTA) freshly made up from a stock 1 per cent solution; that in tube 3 was taken up in 5 per cent formalin. The sediment in tube 4 was suspended in Tyrode's and poured as a shallow layer into a dish that was put in a closed chamber together with a dish of 2 per cent osmic acid for 30 minutes, after which it was transferred to a tube. The suspensions of organisms treated by these various fixatives were spun down, resuspended in distilled water, spun again, taken up in a small volume of distilled water, and transferred to screens. For cultures on solid media, ascitic peptic digest plates incubated anaerobically were used (Smith *et al.*, 1948). All the micrographs were made at 55 kv.

Findings with the 132 Strain

On solid media this organism grows as a rod-shaped bacterium, rather uniform in shape and approximately the size of *Escherichia coli*. Large round bodies and bacillary cells with swellings toward the center or end do occur, but they are not numerous. The remarkable pleomorphism of the organism is best exhibited in liquid media, and the observations here reported were made with cells taken from Brewer's thioglycolate broth and fixed as noted in the legends accompanying the photographs. The pattern of pleomorphism described occurred equally well in meat tubes or in rabbit serum broth incubated in anaerobic jars. The pleomorphism of this strain was best seen in cultures transferred every 24 or 48 hours. Old cultures transferred at long and irregular intervals or kept in the icebox tended to grow as simple rods, rather uniform in size and shape.

The usual method for preparing bacterial cells for electron microscopy has been to suspend them in distilled water. *Bacteroides* cells when suspended in distilled water and transferred to screens gave preparations that were very cloudy; indeed, few cells could be seen in them. The cause for this was not far to seek, and is shown by the following experiment:

Cells from a 9-hour thioglycolate broth culture (figure 1) were washed three times in the centrifuge by spinning them down and resuspending them in Tyrode's solution. The final suspension was divided into two tubes, each of which was spun again. The sedimented cells from one of these tubes (tube A) were taken up in Tyrode's solution; those from the other (tube B) were taken up in distilled water. Within a few minutes there was a sudden clearing of the liquid in tube B, and its contents became slimy so that a gelatinous film was deposited on the glass when the tube was tipped. No such slime formed in tube A, which retained the swirling cloudiness shown by bacterial suspensions for as long as it was kept (5 hours). Obviously, the cells suspended in distilled water had undergone lysis, whereas those suspended in Tyrode's solution had not, as was confirmed by microscopic examination of drops of fluid from both tubes.

This experiment was repeated with four additional broth cultures between 9 and 30 hours old. In the younger cultures the cells were seen as simple rods or as rods bearing swellings upon them. The older cultures consisted almost entirely of large round bodies. When the organisms from these cultures were

suspended in distilled water, they underwent lysis just as had those in the first experiment.   The slime that formed as a result of lysis absorbed so much of the electron beam that it was almost impossible to get clear-cut pictures of the occasional organisms that remained intact.   To make more satisfactory preparations for electron microscopy, drops of organisms suspended in Tyrode's solution were transferred to the screens and permitted to dry, and the screens were then dipped or floated in distilled water to dissolve the salt crystals.   Fewer organisms underwent lysis in these preparations, but the method was far from satisfactory because of the many salt crystals that remained.   A means was therefore needed to make possible the transfer of unlysed organisms to the screens as free as possible of extraneous matter.   It was found that when formalin was added to aliquots of Tyrode's suspensions of these organisms to give a final concentration of 5 per cent then the fixed cells could be spun down and resuspended in distilled water without lysing.   The same preservation of cells was achieved by fixation with phosphotungstic or with osmic acid, and the technique given in the section on "Methods" was therefore devised.

The lysis of these organisms when they were suspended in distilled water suggested the concept, later supported by the micrographs, that their cell walls were more delicate than those of other bacteria; for, with the exception of the organisms of the pleuropneumonia group, other bacteria studied by the electron microscope have been suspended in distilled water without this particular difficulty having been encountered.

The extraordinary pleomorphism of this strain is illustrated in figure 1, which shows organisms from a 9-hour broth culture.   It can be seen that even in such a young and actively growing culture a great percentage of the cells already show swellings and a few large round bodies have formed.   In preceding detailed studies of this strain, it was found that cells from cultures 3 to 6 hours old were mostly regular, uniform rods.   Reproduction during this early phase evidently took place only by simple fission.   By means of the electron microscope, it was possible to show such division by simple fission (figures 3 and 4).   The point is mentioned because of the more complex mode of reproduction next described.

This more complex process began near the ninth hour and was signalized by the development of a bulge or enlargement in the middle or toward the end of the cell (figures 1 and 5 to 11).   It not infrequently happened that this enlargement occurred at the adjacent ends of two cells that had resulted from a recent division by fission but that had not yet completly separated (figures 1 and 5). The bulbous, enlarged areas increased in size until they comprised most of the total cell area, only a small "tail" of the cell retaining the rod-shaped form (figures 10 and 11).   Many cells thus became wholly round (figures 12 to 18), though often retaining a small stump of their original rod-shaped structure (figure 13).

By the twenty-fourth hour the cultures consisted chiefly of these "large round bodies."   They measured 3 to 10 microns in diameter.

The manner of germination of these large bodies was best observed by transferring them to fresh medium.   In previous studies large bodies of this strain were

All figures except 1 and 23 to 26 were made with an electron microscope at 55 kv. The scale on the photographs represents 1 micron.

Figure 1. Cells from a young thioglycolate broth culture. The culture is only 9 hours old, yet the cells already show bulbous enlargements and a few large round bodies have formed. There are several pairs of bacilli bespeaking division by fission. One pair in the middle of the photograph shows enlargement of the adjacent ends. The organisms were placed in a thin film of agar and photographed unfixed and unstained with a light microscope. 1,110 ×.

placed upon a thin layer of anaerobic agar and covered with a sterile cover slip, and photographs were made at hourly intervals with an oil immersion lens (Dienes and Smith, 1944). This procedure provided a cinematographic record of the germination of individual living large bodies. It was thus shown that some large bodies segmented into four or five daughter bacillary cells, whereas others sent out filaments at two to six points on their surface, and these filaments then broke up into bacillary cells. Electron micrographs of such germinating large bodies are given in figures 19 to 22. In figure 22 it can be seen that outpouchings have occurred in six regions of the surface of the body, and it is possible to trace a continuous cell wall enclosing the body and these outpouchings. In other words, the protoplasm of the large round cell has pushed out to form short, twisted filaments extending from the cell but still sheathed with the wall of the cell. The body shown was selected because it shows the successive stages of the production of the new cells. The filament pushing out at the upper left of the body is short and has a rather smooth contour and homogeneous content. The longer filament just below it has a uniform width, as measured by the cell wall, but the protoplasm within the filament has divided into two spherical dense areas. The longest filament, at the bottom of the picture, shows not only the division of the inner protoplasm but also invagination of the cell wall around the spherical protoplasmic masses. From the electron pictures it was thus learned that the new cells produced by the large bodies were derived by simple fission of the multiple filaments that grew out of the bodies.

A striking feature of the cell wall of the large bodies was its veillike delicacy. In most of the photographs the cell wall could not be differentiated. It was visible only where the protoplasm had retracted somewhat from the surface of the cell, as in figure 22. The cell wall of the rod-shaped forms was also very delicate (figure 2) and again could be best identified only where the protoplasm had retracted (figure 6). It appeared as the same type of veillike sheath seen around the large bodies.

Internal Structure of the Cells

The electron micrographs disclosed striking inhomogeneities indicative of segregation within the cells of material with varying ability to scatter the elec-

Figure 2. Rod-shaped cell from 3½-hr culture. The delicacy of the cell wall is apparent. It is seen as a mere veil-like sheath. Osmic acid. 6,100 ×.

Figure 3. Division by simple fission. From the same preparation as figure 2.

Figure 4. Another pair of cells resulting from division by simple fission. Here the process is almost complete. Formalin. 3,300 ×.

Figure 5. A pair of bacilli with enlargement of adjacent ends. Nine-hour culture. Formalin. 4,900 ×.

Figure 6. The cell at left shows beginning of central enlargement in a single cell. In the other cell there has been segregation of the intracellular "dark" material and the veillike cell wall can therefore be seen. Formalin. 5,280 ×.

Figure 7. Three bacilli with enlargements in the middle. A small round cell is superimposed on the bacillus at the left; another lies at the extreme right of the photograph. Toward the lower right corner there is a small, regular rod-shaped cell. It contrasts greatly in size with the large bacilli bearing the swellings. Granules of dark material are seen throughout the cells. They are especially prominent in the central swollen areas. Formalin. 4,900 ×.

Figure 8. Two bacilli with enlargements toward the end. Dark intracellular material is distributed in granules in the enlarged areas of the cells. 15 hours. Formalin. 5,280 ×.
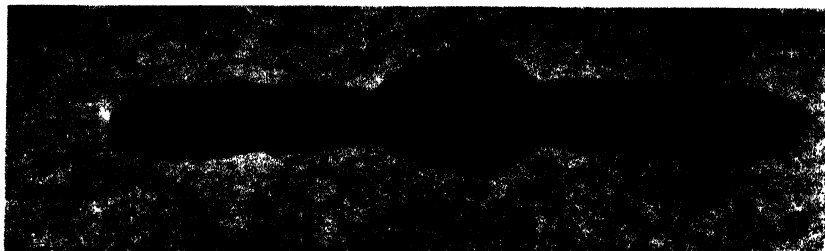
Figure 9. Enlargement of the middle of a cell. The cell membrane is visible in some places as a delicate veil. 9 hours. Formalin. 20,000 ×.

Figure 10. Two cells with enormous, but not uncommon, enlargements. The upper cell still bears the tags of the bacillary cell from which it developed. Bacillary tags are less evident in the lower cell, which is a more fully formed large round body. 15 hours. Formalin. 10,380 ×.

tron beam.   The material that scattered the beam most strongly appeared dark in the photographs.   For convenience it is referred to as the "dark material."

In the rod-shaped cells the dark material sometimes lay in large rounded masses distributed along the course of the cell or in a single mass in the enlarged area of a cell (figure 9).   The dark material was often present in small granules, 0.14 to 0.5 microns in diameter, some of which were scattered throughout the cells, but for the most part these lay in the enlarged areas (figures 7 and 8).

Granules 0.1 to 0.5 microns in diameter were seen in the large bodies (figures 12 and 15).   In some of these the dark material lay in delicately beaded, thread-like strands 0.1 micron wide and 1 micron or more in length (figure 16).   In other large bodies it formed an area occupying much of the cell and showing a well-defined border against the lighter cell protoplasm surrounding it (figures 17 and 18).   We have already described the manner in which the dark material pushed outward in the filaments that extended from germinating large bodies, how it pinched off within these filaments, and how the pinching off of the dark material was followed by invaginations of the wall of the filament to yield new cells, each containing a large rounded mass of the dark material (figures 20 and 22).

The foregoing description of the several patterns in which the dark material was seen could have been made with cells treated with any one of the fixatives employed; in other words, the pattern was not dependant upon the fixative. Certain differences were, however, noted.   Thus, the dark material appeared darker and the surrounding protoplasm lighter in cells fixed by phosphotungstic acid than in cells fixed by formalin or osmic acid.   So sharp was this contrast that phosphotungstic acid seemed a "specific electron micrograph stain" for this material.   In the concentration used, however, phosphotungstic acid made the material appear so black that fine detail within it was lost.   The coarse, twisted threads seen in figure 15 are very similar to those seen in Giemsa-stained organisms.   Fine details, notably the threadlike strands 0.1 of a micron wide, were best brought out by formalin or osmic acid.

It is of interest to compare these electron micrographs with previously published photographs of cells of this same strain of *Bacteroides* stained with Giemsa solution (Dienes and Smith, 1943).   In the Giemsa preparations deeply stained granules were seen within the rod-shaped cells and in some of the large bodies. In other large bodies the deeply stained material lay in coarse, twisted threads or in a large, single mass.   The patterns of the deeply stained material in the Giemsa preparations were, therefore, essentially similar to those of the "dark material" in the electron micrographs.   The inference thus seems justified that the two methods revealed the same material within the cells.   The observation of this material in the unstained cells studied by the electron microscope removed the possibility that the patterns seen in the Giemsa preparations were artifacts of staining.   The finer granules and threads revealed by the much greater resolution of the electron microscope were, of course, not visible in the Giemsa preparations.

Figure 11. Large round body forming at the end of a cell. 15 hours. Formalin. 14,700 ×.

Figure 12. Fully formed large round body. The dark material lies in granules in the equatorial plane and around the periphery. A second cell with an enlargement in the middle lies above the body. 15 hours. Formalin. 10,000 ×.

Figures 13–22. Large bodies in various stages of development. Broth cultures were incubated 24 hours, by which time they had come to consist almost wholly of large round bodies. These were transferred to fresh broth and incubated 3½ to 4 hours in the fresh medium. Screens were then prepared for micrography.

Figure 13. Large body still retaining a part of its bacillary "tail." Finely stippled cytoplasm. Phosphotungstic acid. 6,1000 ×.

Figure 14. Large body from a Tyrode suspension. At least 15 dark granules lie scattered within it. No fixative. 6,100 ×.

L-Type Variation

On solid media this strain formed large colonies up to 5 mm in diameter, comparable to colonies of *E. coli*. When examined by stained wet cutout preparations, such colonies were found to be composed of ordinary large bacillary forms (figure 26), very few of which bore swellings and, rarely, large round bodies. Here and there between these large colonies very different smaller colonies developed (figure 23). These never attained a diameter greater than 1 mm, and in stained preparations they were seen to be made up of small rounded organisms, singly or in twisted chains, and large bodies (figures 24 and 25). The very young colonies of this sort lay burrowed in the agar, but later growth heaped up on the surface of the agar and gave them a "halo" appearance. These differences between the large and the small colonies have been illustrated in previously published photographs (Dienes and Smith, 1944, figures 7, 9, 10). The elements composing the small colonies, their habit of growth into the agar, and their formation of the distinctive surface halo made them resemble very closely the colonies formed by organisms of the pleuropneumonia group (L organisms). They have therefore been called L-type colonies.

L-type colonies rarely developed on plates inoculated with cells from the regular large bacillary colonies or from broth cultures in which large bodies had not yet formed. When, however, cells from large bacillary colonies were transferred to broth and the cultures allowed to grow until large bodies developed in them, subcultures to plates then yielded many L colonies as well as large bacillary colonies. In microcultures photographed at hourly intervals, the L-type of growth was seen to derive from individual, living large bodies. Further, it was possible to influence experimentally the type of germination that took place. Thus, on plates incubated at 37 C, the majority of the large bodies developed into large bacillary cells. On plates incubated at 25 C, the majority of the large bodies underwent the L-type of germination (Dienes and Smith, 1944).

In order to study the germination of large bodies by means of the electron microscope, broth cultures were incubated until they came to consist predominantly of large bodies, and transfers to new tubes of broth were then made. After 4 hours' incubation of the newly inoculated tubes, the cells were spun down and fixed with formalin, and screens were prepared. In this way germinating large bodies were observed. As in the microcultures, many of these extended thick filaments destined to divide into ordinary bacillary cells (figures 19, 20, 22). From some of the bodies, however, very fine, tortuous filaments grew out. These seemed comparable to the large bodies seen undergoing L-type germination in the microcultures. One such body is shown in figure 21. The

Figure 15. Large body with dark material in granules and threads. Phosphotungstic acid. 6,100 ×.

Figure 16. Large body filled with dark material in reticulated pattern. 15 hours. Formalin. 14,700 ×.

Figure 17. A large round mass filling about two-thirds the volume of a large body. This mass contains one prominent large granule but is elsewhere delicately stippled. It seems delimited from the surrounding cytoplasm. Formalin. 6,100 ×.

Figure 18. Another body like that in figure 17. A large, well-delimited mass fills about two-thirds the volume of the body. Osmic acid. 6,100 ×.

Figure 19. Large body with a bumpy, mulberrylike contour. This change in the surface indicates the beginning of germination. Two short, stubby filaments have begun to push out from the body at the top of the micrograph. Formalin. 3,460 ×.

Figure 20. Another germinating large body in which the process is more advanced. Two relatively long filaments have |pushed out from the lower pole of this body, and the body itself has lost the even, round shape. Extensions of the inner dark material have pushed out into the outgrowing filaments. There is some accordion-pleating of the lower filament due to retraction of the parlodion film, shown by the wavy line across the right side of the picture. Formalin. 4,900 ×.

filaments extending from it have a diameter of only 0.3 microns in contrast to the filaments extending from the other bodies in figures 19, 20, and 22, which have diameters of 0.7 to 1 micron. Most of the filaments extending from the large body of figure 21 appeared homogeneous, but segregation of the "darker" components of the protoplasm had occurred in the filament at the upper left of the micrograph. It would thus appear that the germination of some large bodies to yield ordinary large bacillary cells and the germination of others to yield small "L type" variants are essentially a similar process, the difference lying in the size of the filaments extended and the size of the cells produced.

The L-type colonies of this strain of *Bacteroides* have been carried for many transfers on solid media and have preserved their distinctive characteristics. Large colonies composed of the ordinary large bacillary cells never appeared in the transplants. Prolonged cultivation of the L-type variant in liquid media, however, resulted in the reappearance of the large bacillary cells (Dienes, 1948). After several years of cultivation in the laboratory, this strain of *Bacteroides* became steadily less pleomorphic and lost the property of producing L-type colonies. During this time it was found that L-type colonies could readily be got from strains of *Hemophilus influenzae* grown on media containing penicillin or from cultures taken from the respiratory tract when penicillin had been given to patients (Dienes *et al.*, 1948). For this reason the *Bacteroides* strain was plated on media containing penicillin. L-type colonies appeared in great numbers. They seemed wholly to resemble the L-type colonies that had been produced spontaneously during the first years of cultivation of the *Bacteroides*. These L-type colonies preserved their characteristics when they were transferred on solid media devoid of penicillin, but reverted to the large bacillary cells when they were cultivated in broth (Dienes, 1948).

Plate cultures of the L-type colonies induced from the *Bacteroides* by penicillin but carried on media free of penicillin were sent us by Dr. Dienes. In our hands they continued to grow in the L form on transplantation on solid media. Preparations for electron microscopy were best made by picking up tiny 20-hour colonies with a pin and transferring them to a drop of 2 per cent osmic acid on the electron microscope screen. Individual cells could be micrographed at the edge of the colonies in such preparations. Electron micrographs of organisms from these cultures are given in figures 27 to 34.

The electron micrographs disclosed that the small elements in the L-type colonies were in fact tiny round and rod-shaped bacterial cells, averaging

Figure 21. Large body undergoing the L-type of germination. Instead of thick, bacillary filaments, the filaments growing out from this body are narrow and tortuous. Formalin. 3,460 ×.

Figure 22. Germinating large body. Six filaments have grown out from the body and are still sheathed within the cell wall of the body. The dark material in the upper left filament is still homogeneous and continuous with the dark material that fills the large body. In the filament at the lower left, segmentation of the dark material into two rounded masses has occurred. In the filament extending out toward the bottom of the micrograph, the dark material has pinched off into separate masses, and invaginations of the cell wall of the filament have occurred around these masses. Completion of this invagination results in the formation of free daughter cells by simple fission of the filaments. Osmic acid. 17,000 ×.
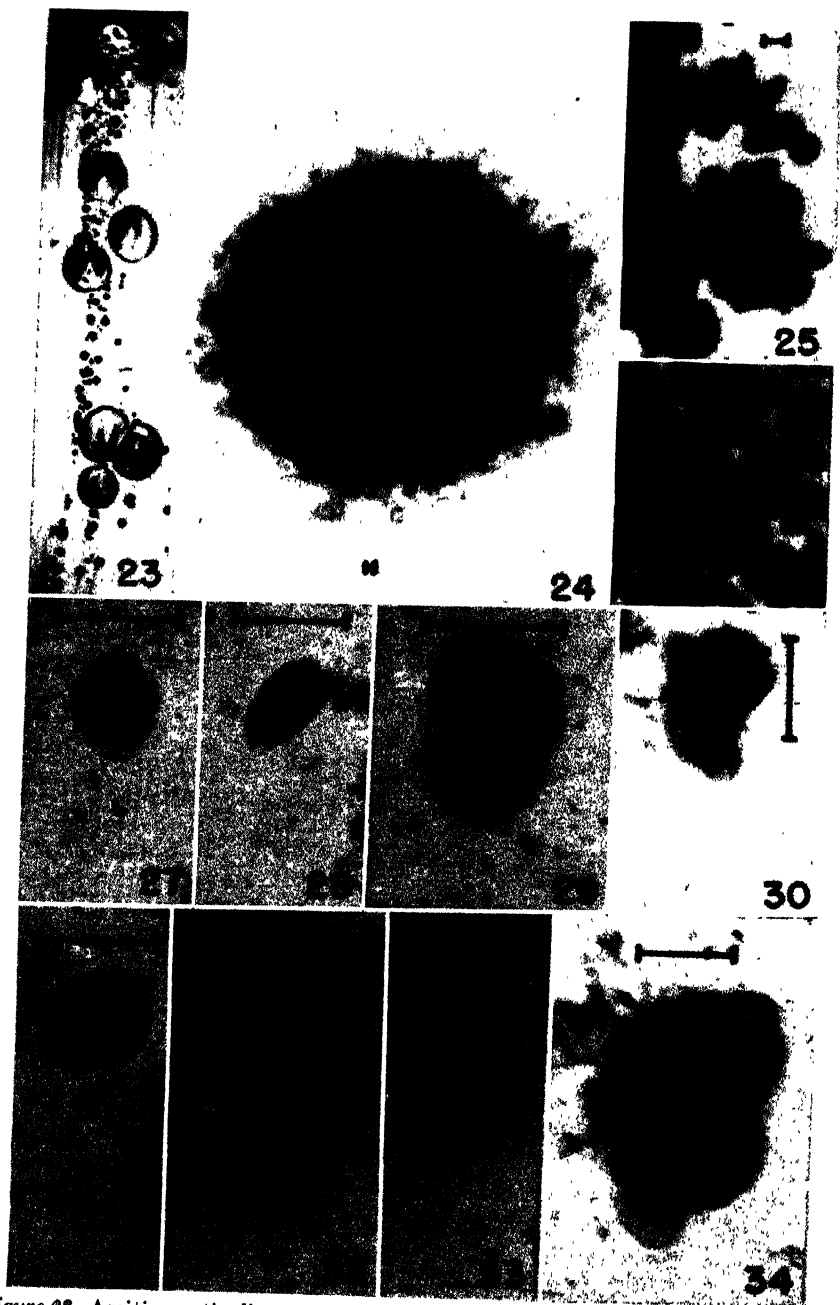
Figure 23. Ascitic peptic digest agar plate 4 days after inoculation from a broth culture of *Bacteroides* in which large bodies had developed.   Note the large bacterial colonies and the tiny colonies of the L-type variant. 1.5 ×. Made with a conventional light microscope.

0.8 microns in size (figures 27 and 28). Larger rod-shaped cells, up to 1.5 microns in length, were also found. These bore bulbous enlargements at one end (figures 29 and 30). It seemed evident that the large round bodies so prominent in the wet preparations arose from such swelling of the cells. The large bodies seen in the micrographs measured 1 to 3 microns in diameter and masses of dark material could be seen within them (figures 31 and 32). Certain of the large bodies exhibited protuberances at several points on their surface that might indicate beginning germination (figures 33 and 34).

The forms seen were thus very similar to those encountered in the electron micrographs of organisms of the pleuropneumonia group (Smith, Hillier, and Mudd, 1948). It will also be noted that, though much smaller in size, the shapes of these forms mimic the pattern of pleomorphism of the large *Bacteroides* cells.

After 24 hours of growth much autolysis took place and the colonies came to consist chiefly of vacuolated large bodies. Electron micrographs made at this time showed much amorphous material, amid which were scattered many extremely minute round or elongated objects of about 0.1 micron in size. These were very opaque to the electron beam, doubtless owing to the osmic acid used to fix the preparations. Some resembled rod-shaped cells with a dark granule at each end, but the micrographs provided no certain evidence that any of these 0.1 micron objects were actual organisms.

DISCUSSION

The electron micrographs confirm the conception of the morphology of the 132 strain of *Bacteroides* gained by previous studies with Giemsa-stained organisms and show that the intracellular granules, large masses, and threads are not artifacts of staining. In addition, much finer granules and threads were found in the electron micrographs than had been visible with light micrography. These finer granules were of the order of size of the microsomes described in animal cells (Claude and Fullam, 1946). Similar fine granules were seen in the cells of the pleuropneumonialike organisms described in the preceding paper.

Figure 24. One of the tiny L colonies as seen in a piece of agar cut out of the plate and examined under a cover slip coated with methylene blue and "azur II." It shows deeply stained granules and large round bodies that stain very lightly. 1,000 ×.

Figure 25. Higher power view of edge of a young L colony before large bodies have become numerous. It is composed of small round organisms, some of which appear elongated. Stained cutout preparation. 3,000 ×.

Figure 26. Edge of one of the large *Bacteroides* colonies examined in same way. It is composed of big bacillary cells of ordinary shape. 2,000 ×.

Figures 27-34. Electron micrographs of organisms from L-type colonies, induced from *Bacteroides* by penicillin. This L variant was carried for 15 generations on media free of penicillin before cells were prepared for micrography. The transplants uniformly showed only L-type colonies. Osmic acid fixation.

Figure 27. Round cell. 16,800 ×.

Figure 28. Rod-shaped cell. It shows a delicate cell wall. 15,600 ×.

Figures 29, 30. Rod-shaped cells that have begun to enlarge at one end. They show that large round bodies develop from the rod-shaped cells of this L variant of the *Bacteroides* in the same manner seen in the large parent *Bacteroides* cells and in the organisms of the pleuropneumonia group. Figure 29 is 19,000 ×. Figure 30 is 14,200 ×.

Figure 31. Large body with two dark masses in its protoplasm. 16,000 ×.

Figure 32. Large body containing masses of dark material. The extreme delicacy of the surface is apparent in the areas between the dark masses. 8,400 ×.

Figures 33, 34. Large bodies with stubby outpouchings indicative of beginning germination. Figure 33 is 14,700 ×. Figure 34 is 13,300 ×.

Of paramount interest were the observations of germinating large bodies. Germination was observed to proceed by the extrusion of filaments from the large bodies. The outgrowing filaments were sheathed with the cell wall of the large body and contained within them masses of the dark material derived from the body. These filaments segmented by simple fission to yield new daughter bacillary cells. Germination was also observed in which large bodies segmented into multiple new daughter cells without marked extrusion of filaments or else with extrusion of only stubby filaments.

The swelling of the bacterial cells to yield large round bodies, much greater in size than the original cells, and the germination of these bodies into multiple daughter cells are very closely similar to the mode of reproduction described for two strains of pleuropneumonialike (L) organisms (Smith, Hillier, and Mudd, 1948). The *Bacteroides* cells, though much larger than those strains, exhibited essentially the same pattern of development.

The thick filaments extruded from *Bacteroides* large bodies segmented into large bacterial cells, but it was known from previous work that certain of the *Bacteroides* large bodies gave rise to very small cells that formed colonies similar in appearance to those of pleuropneumonialike (L) organisms. Colonies composed of these small cells have been called "L-type variants." Large bodies developing in this way were seen in the electron micrographs to germinate by extrusion of multiple filaments, but in their case the filaments were very much thinner. Segmentation of such thin filaments by fission obviously results in cells of a much smaller size.

Electron micrographs were made of organisms from L-type colonies induced by cultivation of this *Bacteroides* strain on media containing penicillin. These showed cells much smaller than those of the ordinary *Bacteroides* but otherwise resembling the pattern of pleomorphism exhibited by the large *Bacteroides* cells.

When the phenomenon of L-type growth was first described, it seemed so very different from the growth of the large bacillary cells that the relation between them was obscure. However, as illustrated in the present paper, large bacterial cells, though ordinarily dividing by simple fission, can also reproduce by the formation of large bodies followed by multipolar germination of these bodies, which is the mode of reproduction distinguishing the pleuropneumonia (L) group. Secondly, as shown in the preceding paper, organisms of the pleuropneumonia group, though ordinarily reproducing by means of large bodies, can also grow as regular tiny bacillary cells. And, in the case of the *Bacteroides* exhibiting L-type variation, it appeared that large bacillary cells resulted when the segmenting filaments extended from the large bodies were thick, that small L-type cells were formed when the filaments were thin. The morphological difference between the large bacillary cells and the L-type variants is thus not as great as it originally appeared. It seems essentially a matter of cell size.

How do these findings bear on the classification of organisms of the pleuropneumonia group? The situation can be summarized as follows: There are certain strains of L organisms that always grow in the small cell, pleuropneumonialike form. These are the strains from cattle, goats, and mice, from rats, and from the human genital tract. Then, in addition, there are the L organisms

that arise as variants in cultures of various species of bacteria, notably *Streptobacillus moniliformis*, *Bacteroides*, *E. coli*, and *H. influenzae*. In the case of *Streptobacillus moniliformis* and *Bacteroides* the variation is reversible: the L variants have been induced to revert to the original large cell size by transfer from solid to liquid media (Dienes, 1939, 1948; Brown and Nunemaker, 1942). In the instances of L-type variation observed in other strains of bacteria, the variation has been irreversible under all conditions thus far studied: the L variants have continued to grow in the small cell, L-type form on transfer. Whether any of the L-strains from the genital tract or from the various animal sources originated by such irreversible variation is not known.

Several years ago the *Bacteroides* strain (no. 132) employed in the present work was sent to Klieneberger. In a recent article she expressed general agreement with our account of its pleomorphism and stated that the L colonies appearing in its cultures were comparable to those of other L strains isolated in her laboratory (Klieneberger, 1947). She made the reservation that final decision as to whether the L colonies were variants of the *Bacteroides* or were symbiotic viruslike organisms associated with it must await demonstration of their bacterial nature and of their reversion to the large *Bacteroides* cells. This evidence now seems to have been provided.

In the present studies it has been found that two strains of pleuropneumonialike organisms (L50 and L4330) and a *Bacteroides* strain that exhibits L-type variation possess a property in common that seems to set them apart from other bacteria. This is the plasticity and fragility of their cell walls. The delicacy of their walls is apparent from the electron micrographs. Further, bacteria examined by electron micrography have usually been suspended in distilled water for the preparation of the screens. This procedure resulted in lysis of most of the *Bacteroides* cells and many of the cells of the two L strains. It seems likely that the plasticity of the cell wall is a considerable factor in accounting for the tendency of these organisms to develop swellings and to assume the shape of large round bodies.

One of us has pointed out (Mudd, 1944) that "evidence is slowly accumulating from many sources to indicate that the specific, pathogenic types of bacteria represent highly differentiated phases which are characteristically found under conditions of active and successful parasitism." The relative rigidity of the cell wall is unquestionably one factor that makes possible the deviation of ordinary bacteria from the spheroidal shape which the naked protoplast would tend to take under the action of surface forces. The minute bacterial cells with plastic cell walls that are characteristic of naturally occurring pleuropneumonialike strains it is tempting to regard as either undifferentiated or dedifferentiated forms, and the minute cells of pleuropneumonialike variants as dedifferentiated forms that have failed to maintain some essential differentiating factor or factors. Incidentally there is much to recommend a similar conception of rough variants as dedifferentiated forms.

The clinical application of L-type variation cannot be evaluated at present. There is evidence that the *Streptobacillus moniliformis* exists in the L form in certain infected human and animal tissues (Brown and Nunemaker, 1942).

This seemed to be the case in the patient from whom the O.H. strain of *Bacteroides* was recovered (Dienes and Smith, 1944). The apparent induction of L variants in patients given penicillin and the resistance of these variants to penicillin should be borne in mind (Dienes *et al.*, 1948).

SUMMARY

Electron micrographs are presented that show a mode of bacterial reproduction differing from that of binary fission, seen in a large anaerobic bacterium, *Bacteroides funduliformis*. Bulbous enlargements develop in the cells and form large round bodies from which multiple filaments grow out. These filaments then segment to yield new cells.

It had previously been noted that certain cultures of this bacterium threw off variant colonies which resembled colonies of pleuropneumonialike (L) organisms and which were called, for this reason, L-type colonies. The minute size of the cells of the L variant had made previous estimation of their nature difficult. A strain of L-type variant induced from this *Bacteroides* by penicillin was examined by electron micrography. It exhibited cell forms similar to those of the large parent bacillus, but smaller in size.

The large *Bacteroides*, its L variant, and the organisms of the pleuropneumonialike (L) group all have in common the ability to reproduce by means of large round bodies.

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THE INTESTINAL MICROFLORA OF HENS AS INFLUENCED BY VARIOUS CARBOHYDRATES IN A BIOTIN-DEFICIENT RATION[1,2]

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The chicken is an animal of great economic importance and is widely used in the assay of certain vitamins, but little is known about its intestinal flora. The first study of the microbial flora of the chicken's intestinal tract was conducted by Kern (1897). His investigations embraced not only the chicken and other gallinaceous birds, but many of the common wild birds. Other work on the microflora of chickens has been reported by Rahner (1901), King (1905), Gage (1911), Menes and Rochlin (1929), and Emmel (1930). All of these workers agreed that *Escherichia coli* and *Aerobacter aerogenes* made up the greatest proportion of the intestinal flora. Other types of microorganisms commonly found in the chicken's intestines by these workers were lactobacilli, micrococci, pseudomonads, bacilli, sarcinae, clostridia (in two instances), and yeasts. Unfortunately, none of these studies was quantitative, and some were not too exact from a qualitative standpoint.

The type of dietary carbohydrate has a marked effect on the intestinal flora of animals. Early studies on the subject are well reviewed by Rettger and Cheplin (1921), who presented further evidence concerning the influence of carbohydrate upon the intestinal microflora. Later studies have confirmed and enlarged upon these earlier reports (Evenson, 1947). From the standpoint of vitamin synthesis, the type of carbohydrate in the diet is an important factor. Elvehjem and Krehl (1947) have reviewed this subject, hence an account of specific reports on the role of carbohydrates upon intestinal biosynthesis of vitamins will be unnecessary. Elvehjem and Krehl state, "One might place the carbohydrates in the following decreasing order in their favorable effect on vitamin requirement: dextrin, starch, lactose, glucose, sucrose, although this varies with different vitamins."

The work presented in this paper was carried out over a period of six months in connection with a nutritional investigation (Couch *et al.*, 1948) that was performed to determine the effects of different carbohydrates upon the intestinal synthesis of biotin by chickens. The microbiological work had as its objective the determination of the influence of various carbohydrates upon the numbers and kinds of microorganisms in the intestinal tract.

<div align="center">METHODS</div>

Animals. The animals employed were Single Comb White Leghorn pullets which had been reared on the experiment station range. Twenty-four pullets were placed in individual battery laying cages equipped with raised screen bottoms in order to minimize coprophagy. An all-mash laying ration was fed these hens 4 weeks prior to the start of the experiment. The pullets were divided into six groups of four each, and placed on six different diets, five of which were composed of purified ingredients (Couch *et al.*, 1948). The five synthetic rations were numbered B31, B32, B33, B34, and B35. They contained as their sole carbohydrate sucrose, dextrin, lactose plus sucrose, sucrose, and whey plus sucrose (the weight of the whey was adjusted to give the same percentage of lactose as in B33), respectively. Ration B34 contained added biotin and was considered nutritionally adequate; the other diets were biotin-deficient. The sixth group of birds was fed the same all-mash laying ration that they had received during the pre-experimental 4-week period.

Concurrent with the bacteriological work, Couch and collaborators made a nutritional study of the pullets. They kept an account of the egg production and hatchability, the abnormalities of the embryos and hatched chicks, the weekly weight gains of the hens, and the biotin content of the yolks and whites of some of the eggs produced.

Microbiological determinations. Fecal droppings for bacteriological analyses were collected on large pieces of clean waxed paper which were placed beneath the raised screen bottoms. As soon as a dropping was deposited upon the paper, it was removed with an alcohol-flamed spatula and put in a sterile wide-mouthed bottle. All fecal samples were collected between the hours of 7:45 and 9:30 A.M.

As soon as four to six samples were obtained (usually within a 0.5-hour period), the feces were prepared for analyses. One-half gram of each sample was weighed to the nearest milligram on a small piece of clean waxed paper and placed in a 49.5-ml (sterile tap water) 6-ounce dilution bottle containing a half-inch layer of glass beads. These initial 1:100 dilutions were kept on ice for a short time until preparations were completed for their microbiological analyses. The first dilutions were vigorously shaken until all visible clumps were dispersed. Serial decimal dilutions were then made in sterile tap water up to a 10^{-9} or 10^{-10} dilution and inoculations made into the following media: (1) 0.5 per cent tryptone, 0.3 per cent yeast extract, 0.5 per cent glucose, 1.5 per cent agar for aerobic plate counts; (2) "Thioglycolate medium" (Baltimore Biological Laboratories) for total dilution counts (steamed and cooled prior to inoculation); (3) carrot-liver extract ("CL") agar shake tubes (Garey *et al.*, 1941) for an indication of numbers of lactic acid bacteria; (4) "SF" broth of Hajna and Perry (1943) for dilution counts of enterococci; (5) Difco eosin methylene blue agar ("EMB") for coliform plate counts; and (6) potato-glucose agar acidified to pH 3.5 with citric acid (according to *Standard Methods for the Examination of Dairly Products*, 1941) for the enumeration of yeasts. Cultures prepared with media (1), (2), (3), and

(5) were incubated at 37 C; those with medium (4) were incubated at 45 C; and medium (6) cultures were incubated at room temperature. Cultures prepared with media (3) and (4) were incubated for 3 days, those with medium (6) for 5 days, and cultures made with other media were incubated for 2 days before counts were made. Five tubes per dilution were used for all dilution counts, and the most probable numbers of organisms in these samples were determined from the table in *Standard Methods for the Examination of Water and Sewage* (1946). All plates and shake tubes were run in duplicate. Dry weight determinations were made of each sample so that the final count could be expressed on a dry weight basis; this eliminated some of the variations encountered.

Occasional gram stains were made from smears of the 1:100 dilution and examined. No direct counts were made, however, since it was felt that such counts would contribute little to the objective of this investigation.

At the conclusion of the 6-month nutritional study it was possible to sacrifice the animals in order to obtain information on the distribution of the microorganisms within different segments of the intestinal tract. Two hens from each ration group were sacrificed by decapitation, and a posterior ventral incision was made so that the entire intestinal tract could be laid out. Representative 0.5-g samples were removed from the duodenum (a mixture of ascending and descending loops), ileum (from the middle 6 inches), cecal pouches, and colon. These samples were handled in the manner already described for fecal droppings, except that dry weight determinations were not made because many of the samples were insufficient in amount for such a purpose after the aliquot for microbiological procedures had been obtained.

Statistical analysis. Because of extreme variation in the results secured, the data on fecal dropping counts were analyzed statistically according to Snedecor (1946) and Torrie (1948). The data secured from samples taken at different levels of the intestinal tract were believed inadequate for statistical treatment.

For convenience in handling each individual count was converted to its respective logarithm to the base 10 (the mantissa was carried out to the second place). An analysis of variance for counts obtained on each medium was made, and the F value was determined. Since the coliform flora of hens on ration B31 was so obviously different from that of hens on the other five rations, EMB counts of feces from these four pullets were omitted from the analysis of variance in order to allow a more valid test of significance between such counts from hens on the remaining rations. The following statistics were then calculated: (1) arithmetic mean ($\bar{x}$); (2) standard deviation (s); (3) standard error of the mean ($s_{\bar{x}}$); (4) coefficient of variation ($C. V.$); and (5) least significant difference (LSD) where the F value was significant at the 1 or 5 per cent level of probability.[4]

In plotting the data graphically, it was decided to use modal values of fecal

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[4] Note that all statistics are computed from logarithms, hence their antilogarithms will not be exactly similar to like statistics calculated from natural numbers of individual microbial counts. However, this more convenient method will result in comparable values, i.e., the change will be proportionate (Torrie, 1948).

counts rather than arithmetic means.   It was believed that modes would give a more representative picture for comparative purposes since extreme values would be eliminated.

RESULTS

Fecal droppings.   The enumerative results of this portion of the experiment may be seen in table 1 and figure 1.   Note the similarity of the agar plate and Brewer's dilution counts on feces of birds on the six rations.   Differences in fecal counts between hens on the various diets appear to be most prominent in carrot-liver agar shake (CL), SF dilution, eosin methylene blue agar plate (EMB), and yeast counts.   The two lactose-containing diets (B33 and B35) appear to stimulate the development of lactic acid and coliform bacteria as indicated by their CL and EMB counts.   Ration B31, which contained sucrose as its sole carbohydrate, was very depressing to coliforms.   Enterococci appeared to be favored most by the grain and B35 rations.   Dextrin (ration B32) favored intestinal coliforms but was somewhat depressing to yeasts.

Analyses of variance for the counts on feces of hens made with the six media indicate that differences between counts from birds on the six rations (five for coliforms) are highly significant (the probability, P, of assuming a significant difference is 0.01 or greater, or, 1:100) in the case of CL, SF, and yeast counts; the differences were only significant ($P = 0.05$) for fecal counts of coliform organisms.   Examination of table 2 reveals a high degree of variation between individual counts in any one ration group (within rations variation) for coliform determinations, as indicated by the high value (.246) for s.   Also, the $C. V.$ for coliforms is very high (55.09 per cent), which would tend to throw some doubt upon the validity of the EMB F value.   The remaining $C. V.$ values were fairly similar; $s_{\bar{x}}$ values were comparable except in the case of coliform counts.

From results of LSD calculations, CL counts on feces from hens on rations B33 and B35 were highly significantly different from the rest, but not from each other; no significant difference was found to exist between the four low-count CL enumerations.   Enterococcus counts revealed two rations which yielded droppings of comparatively high counts: B35 and grain.   Fecal SF counts from hens on these two rations were not significantly different from each other or from samples of B34-fed hens.   However, they were highly significantly different from SF counts on feces of hens on ration B31, and significantly different from B32- and B33-fed pullets.   The LSD of SF counts between rations B31 and B34 was significant, but not between B32- and B34-fed birds.   Despite the wide differences shown in fecal coliform counts, the F value was significant only at the 5 per cent level of probability.   Since EMB counts of samples from animals fed ration B31 were left out of the analysis of variance for coliforms, LSD's between B31-fed chickens and any of the other ration groups could not be calculated.   However, its difference of greater than 2.5 logarithms from the next lowest group (grain) would indicate that these counts differ highly significantly from coliform counts obtained from hens on any one of the other five diets; most of the $LSD_{.01}$'s for coliforms ranged from 2.2 to 2.3 + logarithm units.   The two high-count coliform groups (B32 and B33) gave an LSD value which was not

TABLE 1

Summary of microbial counts of fecal droppings from hens

MEDIUM	RATION	AVERAGE	MODE	NO. OF SAMPLES
CL		(10^6)*	(10^6)	
	B31	6,675.0	4,160.0	18
	B32	3,684.0	3,199.7	17
	B33	109,014.4	128,777.0	13
	B34	2,936.6	2,548.5	17
	B35	52,225.8	49,084.0	13
	Grain	6,504.6	3,134.3	17
Brewer's dilutions		(10^6)	(10^6)	
	B31	2,573.0	1,550.8	16
	B32	1,559.2	2,012.4	16
	B33	1,453.1	929.5	12
	B34	1,603.2	897 7	15
	B35	4,812.7	1,604.0	11
	Grain	1,395.4	1,138 4	18
SF		(10^4)	(10^4)	
	B31	107.1	123.5	7
	B32	534.7	293.6	8
	B33	819 2	561.7	7
	B34	1,747.9	787.6	9
	B35	6,758 4	2,651.5	7
	Grain	4,348.6	2,469.3	8
Agar plate		(10^5)	(10^5)	
	B31	4,082.1	1,875.0	20
	B32	2,331.0	2,003.0	18
	B33	2,287.6	1,873.7	14
	B34	1,840.1	2,532.8	17
	B35	5,755.0	3,042.1	13
	Grain	10,127.4	5,159.0	19
EMB		(10^4)	(10^4)	
	B31	0 06	0.01	20
	B32	4,078.4	1,210.2	18
	B33	2,592.1	839.9	14
	B34	1,328.0	6.5	17
	B35	397.6	64.8	12
	Grain	78.9	3.5	18
Acidified potato-glu-cose agar		(10^3)	(10^3)	
	B31	2,785.0	2,785.0	7
	B32	382.8	16.6	7
	B33	3,994.4	3,163.1	6
	B34	4,896.0	3,847.0	7
	B35	5,160.5	3,386.6	6
	Grain	99.7	169.6	7

* All counts are expressed in numbers per gram of dry weight; the average moisture content was about 80 per cent.

significant.   Neither B32- nor B33-fed birds yielded fecal droppings which were found to differ significantly in numbers of coliforms from those of chickens fed
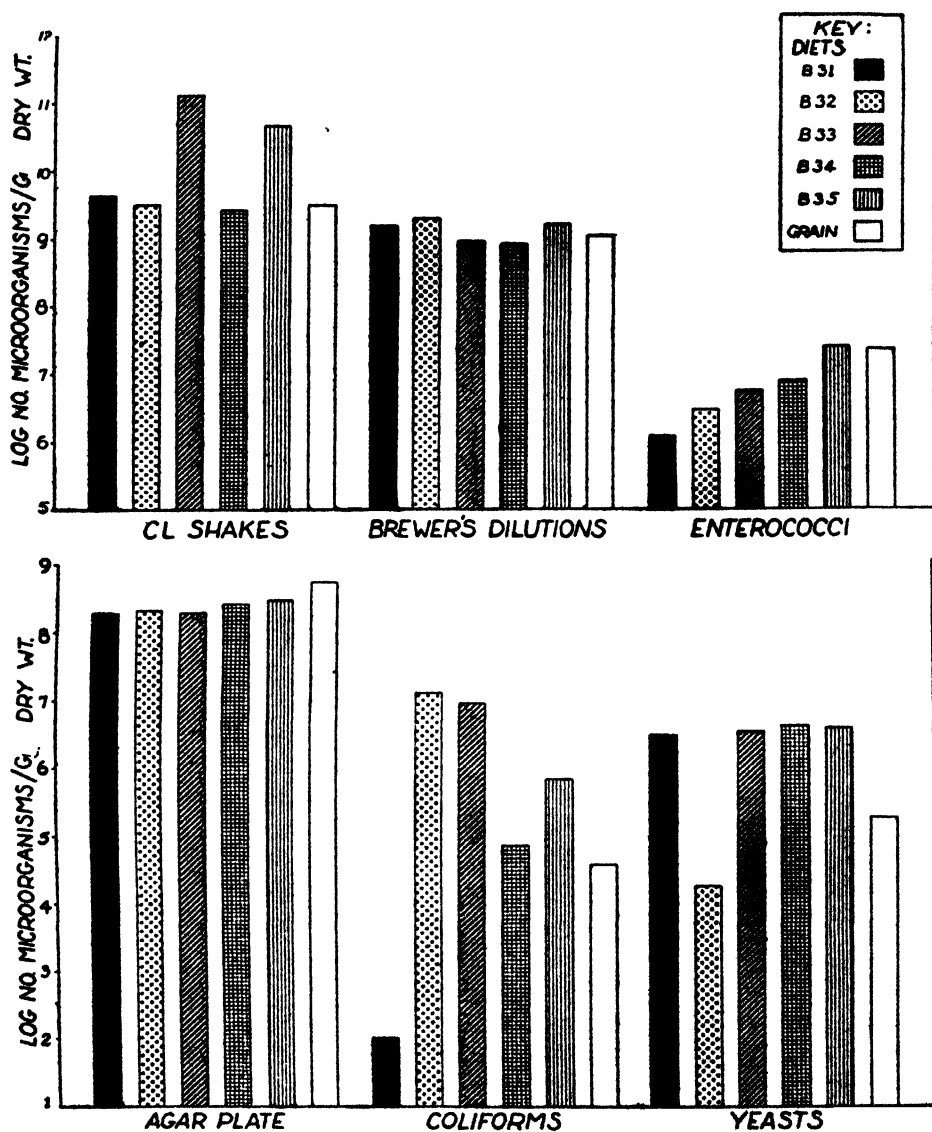
Figure 1. Fecal counts (modal values) from hens on various rations.

B35, but their *LSD*'s were significant when compared with B-34 hens.   The following comparisons resulted in the finding of no significant differences in numbers of fecal coliforms: B34 and grain; B35 and grain; and B34 and B35. The grain and B32 rations were found to be least stimulatory to the growth of

yeasts. *LSD* calculations revealed that the difference between yeast counts of feces from hens fed the latter diets was not significant, and that fecal yeast counts on hens fed rations B31, B33, B34, and B35 were not significantly different from one another. However, the feces of chickens on any of the latter four rations had average yeast counts which differed significantly from those fed B32 or grain.

Difficulty was encountered in isolation of lactobacilli from the fecal samples, but enterococci could be isolated with ease from CL shakes and SF dilution tubes. Numerous gram stains of representative colonies appearing in CL tubes revealed cells morphologically similar to lactobacilli along with large cocci. Therefore, it was believed that CL counts, particularly at the higher dilutions, rep-

TABLE 2

Summary of statistical analyses of enumerative data from fecal droppings of hens on various rations

RATION	TYPE OF COUNT					
	Coliforms	Yeasts	Agar plate	Brewer's	Enterococci	CL agar shakes
B31	—	6.27[1]	8.10	8.78	5.76	9.35
B32	5.35	3.84	8.06	8.97	5.96	9.33
B33	5.55	6.35	7.88	8.59	6.16	10.58
B34	3.39	6.49	7.97	8.71	6.66	9.03
B35	4.74	6.55	8.33	9.06	7.42	10.21
Grain	3.56	4.40	8.30	8.44	7.07	9.33
STATISTICS[2]						
$\bar{x}$	4.46	5.61	8.11	8.75	6.51	9.58
s	2.46	1.02	0.73	0.84	0.88	0.86
$s_{\bar{x}}$ . .	0.28	0.16	0.07	0.09	0.13	0.09
C. V.	55.09%	18.20%	8.94%	9.64%	13.57%	8.99%
F	2.725*	9.404**	0.944	1.124	4.040**	7.095**

[1] Average logarithm of the logarithm for each individual count.

[2] $\bar{x}$ = arithmetic mean; s = standard deviation; $s_{\bar{x}}$ = standard error of the mean; C. V. = coeficient of variation; F = F value (*–F significant at the 5 per cent point; **–F significant at the 1 per cent point).

resented a fairly accurate enumeration of lactic-acid-producing bacteria in the feces. When colonies appearing in such shake tubes were picked and transferred into CL broth, the gram-positive, rod-shaped bacteria rarely "came up" after incubation for as long as one week at 37 C. The cocci grew in nearly all cases. The few lactobacilli which were isolated were anaerobic to microaerophilic, and reduced litmus, produced acid, and coagulated skim milk within 24 to 36 hours at 37 C. Because of the difficulty in isolating and maintaining these lactobacilli, no further study was made of them. The enterococci were not studied to any great extent, but those encountered in the feces of young chicks have been partially characterized and found to be *Streptococcus faecalis* (Johansson and Sarles, 1948).

Coliform bacteria appeared to be predominantly of the *E. coli* and inter-

TABLE 3
Summary of microbial counts at four levels of the intestinal tract

MEDIUM	LEVEL OF INTESTINE				
	Ration	Colon	Cecum	Ileum	Duodenum
CL		(10^6)	(10^6)	(10^6)	(10^6)
	B31	220	3,950	36.2	11.5
	B32	650	5,150	113.0	4.9
	B33	626	8,500	31.2	12.0
	B34	280	905	35.0	10.4
	B35	1,567	2,100	17.7	4.7
	Grain	1,725	14,000	12.5	0.4
Brewer's dilutions		(10^6)	(10^6)	(10^6)	(10^6)
	B31	192	2,950	28.0	79.0
	B32	721	6,350	185.0	9.3
	B33	54	177	24.9	12.0
	B34	262	560	9.0	0.2
	B35	2,315	1,140	1.2	0.9
	Grain	250	4,770	1.9	0.3
SF		(10^4)	(10^4)	(10^4)	(10^4)
	B31	1,100.0	239.0	280.0	0.02
	B32	2,300.0	350.0	300.0	18.7
	B33	—	—	—	—
	B34	—	—	—	—
	B35	57.0	4.1	80.0	17.5
	Grain	2.8	259.0	4.8	3.3
Agar plate		(10^5)	(10^5)	(10^5)	(10^5)
	B31	665	242	157.0	37.0
	B32	4,800	4,620	5,070.0	80.0
	B33	11	25	1.2	0.5
	B34	688	119	160.0	36.0
	B35	450	191	31.1	6.8
	Grain	90	490	145.0	4.0
EMB		(10^4)	(10^4)	(10^4)	(10^4)
	B31	0.008	0.55	0.008	0*
	B32	37.59	578.5	6.2	0.24
	B33	1.97	23.61	0.001	0.008
	B34	0.008	0.04	0.01	0.01
	B35	214.0	50.0	0.007	0.005
	Grain	0*	0.68	0*	0*
Acidified potato-glucose agar		(10^3)	(10^3)	(10^3)	(10^3)
	B31	80	15.0	94	8.8
	B32	2,220	287.0	4,600	59.0
	B33	103	16.0	10	10.0
	B34	4,200	137.0	53	149.0
	B35	460	10.7	111	19.0
	Grain	5	8.2	1	0.05

Each count is the average of two samples and is given on a fresh weight basis.
* Count of 0 means no colonies at the 10^{-2} dilution.

mediate types; only an occasional typical *A. aerogenes* colony was observed on EMB plates from any fecal sample.
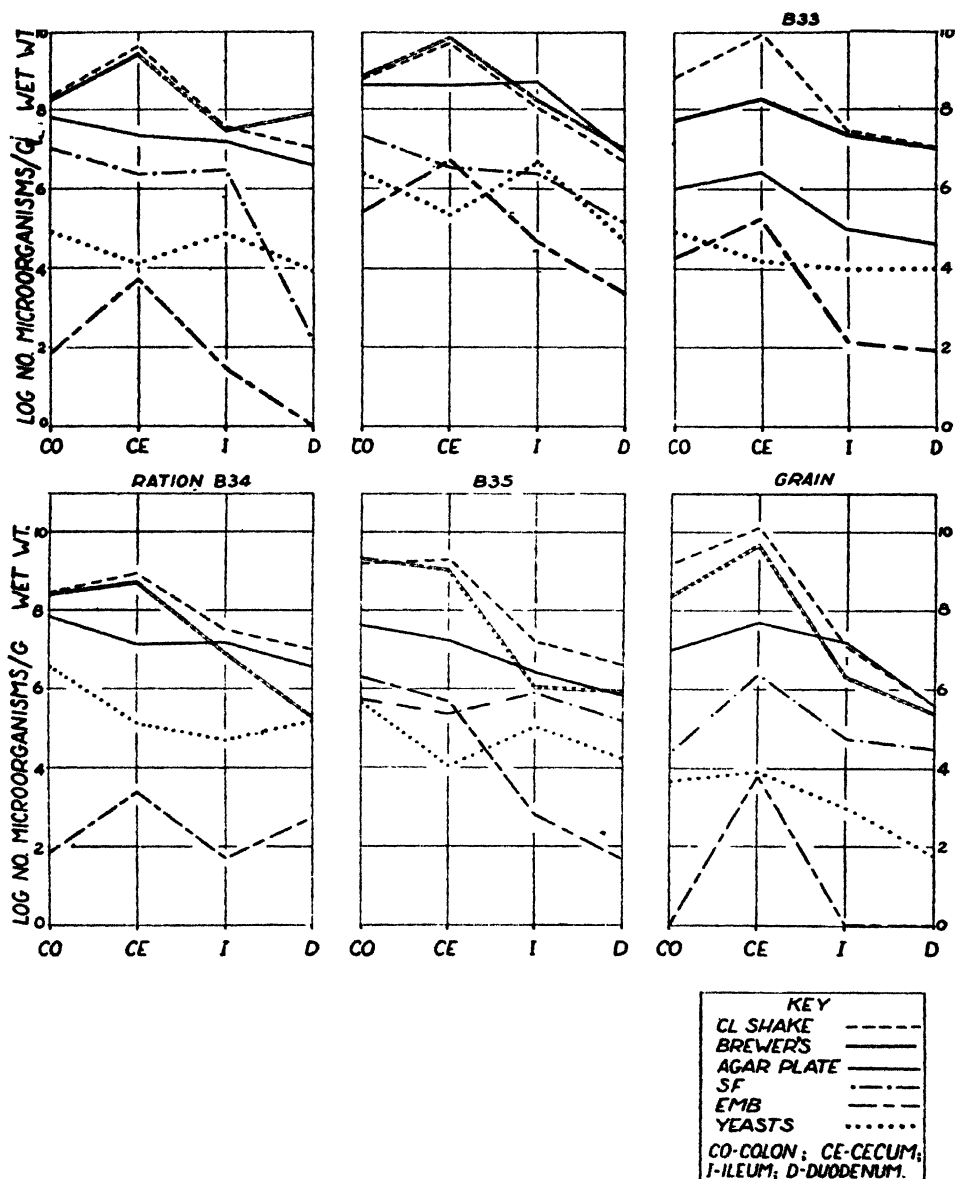
Figure 2. Intestinal flora at different levels from hens on various rations.

Seventy yeast cultures were isolated from the acidified potato-glucose agar plates. All grew well at 37 C, 14 produced a coral red pigment, 13 fermented dextrin, and 2 fermented lactose. However, those isolates able to ferment

lactose or dextrin did not necessairly come from fecal samples of birds consuming a lactose or dextrin diet.

It is believed that Brewer's dilution counts gave a fair indication of the anaerobic flora in fecal samples. Frequently, some of the highest dilution tubes evidenced growth indicative of a pure culture. Pleomorphic, gram-positive rods were isolated from such tubes but grew with difficulty and soon were lost.

Intestinal contents. Inspection of table 3 and figure 2 reveals an over-all increase in numbers of bacteria from the duodenum to the colon, and points to the cecum as the seat of the greatest concentration of microorganisms. In every case, CL counts reached a peak in the cecum and abruptly decreased between the cecum and duodenum. A difference in CL counts of intestinal contents between the lactose-fed hens and those from hens on the other rations was not apparent despite the fact that marked differences had been noted in the fecal droppings from these birds. However, on the basis of two samples for each ration, a difference is difficult to show, and might not be statistically significant even if it appeared to be obvious. Brewer's dilution counts paralleled CL counts at a lower level except in birds fed B32; in them Brewer's counts were slightly higher than CL counts.

Enterococcus counts were not obtained from hens on rations B33 and B34 because of incubator troubles. Only in birds on diet B31 was there a striking drop in numbers of enterococci from the colon to the duodenum. This decrease is but slight in the tract of hens fed B32; birds on B35 or grain showed no such decrease. Agar plate counts generally followed those obtained with CL and Brewer's media, but at a lower level, and with less differences between segments of the tract.

Coliforms increased in numbers going down the intestinal tract (from the duodenum to the cecum) except in hens on the "complete" synthetic ration (B34); in these birds coliforms were present in almost as great numbers in the duodenum as in the cecum, but the total numbers were comparatively low. No coliforms were detected in the duodenum of hens on B31, nor in the colon, ileum, or duodenum of hens on grain. Hens on dextrin (B32) maintained the richest flora of coliforms at all levels, whereas B31- and grain-fed birds possessed meager populations of these organisms.

Yeasts were found at all levels of the intestinal tract of hens on each ration. Only a very slight increase was found in samples from the duodenum to the colon. It is interesting to observe that pullets fed the dextrin ration had considerably more yeasts in their intestines than were found in fecal dropping samples. Only in hens fed the all-mash diet did yeast counts drop below 10^4 per gram at any level.

DISCUSSION

Fecal droppings. Several differences between the fecal flora of hens on the six rations might be considered important: (1) the extremely low numbers of coliforms in samples from hens on the sucrose-containing ration B31; (2) the relatively low numbers of yeasts in droppings from birds on the dextrin ration (B32);

and (3) the very high carrot-liver extract agar shake tube counts on fecal samples of pullets on rations B33 (lactose + sucrose) and B35 (whey + sucrose). Other differences were statistically significant, e.g., enterococcus counts and those between coliform counts of hens on rations other than B31 (sucrose).

The factor which interfered most with this study was that of variation between individual counts from hens on any one ration; even between counts from the same hen from week to week the variation was considerable. For example, the lowest coliform count obtained from hens on ration B32 was less than 100 per gram, whereas the highest was $49,200 \times 10^4$ per gram, dry weight (average $= 4,078.4 \times 10^4$; mode $= 1,210.2 \times 10^4$). Thus, statistical treatment of these data was necessary. The reason for such extreme variation is not known. At first it was believed that differences in water content of the fecal samples might increase the variation; hence all counts were expressed on the dry weight basis. This reduced the variation somewhat. A further step was instituted, and counts were determined on the basis of the dry organic matter content of the samples. This failed to alter appreciably the variation. A more likely explanation lies in the source of the fecal dropping taken for analysis. Cecal pouch contents are usually evacuated in the morning shortly after sunrise, and in the evening shortly before sunset. Therefore, a cecal dropping, which has a characteristic appearance, was not uncommon at the time of collecting (around 8:00 A.M.). The cecum appears to be the site of the greatest concentration of intestinal microorganisms. Therefore, a cecal sample, or a mixed cecal sample, would be expected to deviate in its microbial content from that of a usual colon sample. It is questionable that this can account for all of the variation observed. Other factors probably are involved, e.g., the time of day, time elapsed since last feeding, individual variation between birds under identical conditions, and experimental error.

Least significant differences, which were calculated only from counts on those media showing significant F values, give an indication as to the degree of significant difference that can be detected with the various media employed. For instance, at the 5 per cent point, the difference in coliform counts between samples from hens fed the six diets had to be approximately log 1.8 (nearly a 100-fold difference) to be considered significant. It was found, thus, that unless a difference is anywhere from 8-fold (for CL counts) to 100-fold (for EMB counts), it cannot be detected by the methods employed in this study.

The manner in which the data for fecal droppings were handled does not permit an inspection for trends. Only one definite trend was observed: a gradual increase in numbers of yeasts in feces from hens on ration B32 (dextrin) at the close of the study. At one point during this increase a tremendous development of red-pigmented yeasts was noticed, almost to the exclusion of the more common white varieties. However, the predominance of pigmented yeasts soon subsided to a point at which their occurrence was more sporadic, as found in the other samples, but the yeast flora of these dextrin-fed hens then remained at the higher level (note the high level of yeasts in the various levels of the tracts of these birds).

The results of Couch *et al.* (1948) suggested that the only birds receiving sufficient biotin, either by ingestion or biosynthesis, were those on rations B32, B34, and grain. Therefore, it must be concluded that the carbohydrate, dextrin, exerted a pronounced stimulatory effect upon microbial synthesis of biotin. The dextrin-fed hens possessed very high numbers of coliform organisms in their fecal droppings; coliforms are notably good biotin synthesizers (Landy and Dicken 1941; Landy *et al.*, 1942; Thompson, 1942; Burkholder and Mc-Veigh, 1942). Also, these chickens were found to have relatively small numbers of fecal lactic acid bacteria, which are known to have high requirements for B vitamins. One might therefore conclude that dextrin encouraged the development of a biotin-synthesizing intestinal flora and discouraged the proliferation of a biotin-utilizing flora.

The stimulatory nature of dextrin on the synthesis of B vitamins in other animals is well known, but sucrose and lactose have usually been found to be of no particular value in establishing a vitamin-synthesizing flora in various experimental animals on purified rations (Elvehjem and Krehl, 1947). Hens fed lactose (B33) or whey (B35) also had a rich fecal coliform flora. However, they were found also to have large numbers of lactic acid bacteria. A competitive microbial equilibrium for biotin might have existed in these hens. In the case of whey, the minerals as well as lactose might have exerted some influence upon biotin-synthesizing bacteria (Daniel and Harvey, 1947). Sucrose was extremely depressing to coliform organisms; the majority of determinations resulted in the finding of fewer than 100 coliforms per gram in fecal droppings from these hens. It is difficult to understand why sucrose-fermenting coliforms such as *A. aerogenes* and *E. coli* var. *communior* did not establish themselves in these birds.

Pullets on the grain and B34 rations appeared to have a more balanced fecal flora, i.e., no one group of microorganisms was strikingly high or low in numbers.

There is already fair evidence of biotin synthesis in animals (McElroy and Jukes, 1940; Wegner *et al.*, 1941; Nielsen *et al.*, 1942; Mitchell and Isbell, 1942; McGregor *et al.*, 1947); and in humans (Gardner *et al.*, 1943, 1945, 1946; Oppel, 1942). It can be assumed, then, that the hens on the purified biotin-deficient diet containing dextrin as the sole carbohydrate were able to synthesize and assimilate adequate amounts of biotin; the source of such biotin is apparently from metabolic activities of intestinal microorganisms, perhaps those belonging to the coliform group.

One unknown quantity is that of strain difference among biotin-synthesizing microorganisms. Chickens kept on the two lactose-containing diets may have had few strains of coliforms able to synthesize appreciable amounts of biotin. Chemical and biological antagonisms should be considered also when evaluating the over-all influence of the intestinal flora on the nutrition of the host.

The carrot-liver extract agar of Garey *et al.* (1941) was by no means selective. However, lactic acid bacteria were present in such large numbers that, in effect, a lactic count was obtained as the result of dilution. The difficulty in isolation of lactobacilli might be indicative of large numbers of the anaerobic type, *Lacto-*

bacillus bifidus. It is not known whether the Brewer's medium dilution counts indicated actual numbers of anaerobic bacteria in the samples except that anaerobes could be isolated from this medium. Agar plates incubated aerobically also provided little qualitative information; colonies of sarcinae, micrococci, actinomycetes, bacilli, coliforms, and other types of microorganisms were encountered; it is doubtful that lactobacilli developed in these plates as prepared.

Intestinal contents. A brief survey of these data reveals increasingly greater numbers of intestinal microorganisms from the duodenum to the cecum with a slight decrease from the latter segment to the colon. This agrees with the early work on the intestinal flora of chickens and other birds (Rahner, 1901; King, 1905; Gage, 1911).

The duodenum of all hens studied contained very few coliform organisms. In hens on rations B31 or grain no coliforms were found in the 10^{-2} dilution of duodenum contents (recorded as zero). Yeasts were found in relatively large numbers in the duodenum of all birds except those consuming the grain mash, and increased the least of any of the organisms involved in this study from the duodenum to the colon. Enterococci also were encountered in relatively high numbers in the duodenum; these results agree to some extent with those of Kendall and Haner (1924), who claimed that *Micrococcus ovalis* (*Streptococcus faecalis*) is present in the largest numbers in the duodenum of the human intestine. Agar plate, Brewer's dilution, and CL counts indicated approximately the same concentration of microorganisms in the duodenum.

The flora of the ileum was very similar to that of the duodenum except that it appeared to contain slightly greater numbers of bacteria. No coliforms were found in the ileum of hens on the grain diet.

Colon counts were somewhat lower than cecal counts and slightly higher than ileum counts. The importance of the colon in the chicken in relation to intestinal synthesis is uncertain. It is a very small segment of the intestinal tract wherein fecal material resides for but a brief time before evacuation. Vitamins elaborated in this segment may be very incompletely absorbed by the host before being passed out of the tract. On the other hand, the cecum, which in the avian species has two large lobes, is a reservoir which is emptied usually twice a day; it may thus be of great importance in the synthesis and absorption of biotin as well as other B vitamins. Cecal counts were found to be the highest of any segment of the tract in this work. This is particularly true in the case of CL, Brewer's dilution, and coliform counts. Coliforms were found only in the ceca of birds on grain. The latter finding may have a bearing on the role of the cecum in intestinal synthesis because of the ability of coliforms to synthesize a wide variety of B vitamins, including biotin.

Birds on diet B32 (dextrin) generally had the greatest numbers of microorganisms at all levels of the intestinal tract as well as the least "spread" between the different counts. The latter might be indicative of a more heterogeneous flora in these birds.

It was hoped that the study of the microflora at different levels of the intestines

of these hens would yield results that might lend themselves, in part at least, to an explanation of the findings of the biochemists (Couch *et al.*, 1948). It is believed that the high concentration of bacteria in the cecum points to this organ as the focus of intestinal B vitamin synthesis, and we might assume that biotin was synthesized in the ceca of hens on the dextrin-containing ration. Several good studies on the rat indicate, in fact, that the cecum is the site of the greatest synthesis of B vitamins: Guerrant *et al.*, 1935; Mitchell and Isbell, 1942; Taylor *et al.*, 1942; Day *et al.*, 1943; McGregor *et al.*, 1947. The ingenious method of Mitchell and Isbell revealed the rat's cecum to absorb the greatest proportion of the various B vitamins synthesized therein by bacteria. Is the chicken similar to the rat in this respect?

Digestive enzymes might be intimately concerned with the stimulatory nature of dextrin and the depressing effect of sucrose and lactose toward biosynthesis of vitamins. Dextrin may be incompletely attacked by digestive enzymes, thus permitting a residual amount of fermentable carbohydrate to reach the cecum; this carbohydrate may be vital for vigorous vitamin synthesis. Sucrose and lactose, which are relatively soluble disaccharides, may be completely assimilated by the time peristalsis brings the ingesta into the cecum, and the consequent lack of carbohydrate may decrease the vitamin-synthesizing activities of cecal organisms.

SUMMARY AND CONCLUSIONS

The type of dietary carbohydrate was found to influence the microflora of fecal droppings from laying pullets ingesting a purified biotin-deficient ration. Dextrin was found to stimulate the development of considerable numbers of coliform bacteria. Lactose-containing diets likewise encouraged a fecal coliform flora, but lactic acid bacteria proliferated extensively in the intestines of hens fed such a diet. The most marked effect noted in case of the sucrose diet was a depressing action on fecal coliforms.

In general, intestinal microorganisms were found to increase in numbers from the duodenum to the cecum; yeasts and enterococci increased the least. Birds on the dextrin-containing diet appeared to have the greatest numbers of microorganisms at all levels of the intestinal tract as well as the least "spread" between the different counts. The cecum was found to be the site of the greatest concentration of intestinal microorganisms.

An analysis of variance was successfully applied to the data from fecal dropping samples. This revealed the presence of certain differences that were further detected by least significant difference calculations.

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BACTERIAL POPULATION CHANGES IN THE CECA OF YOUNG CHICKENS INFECTED WITH EIMERIA TENELLA[1,2]

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Eimeria tenella, a protozoan parasite belonging to the class *Sporozoa*, is the etiological agent of cecal coccidiosis of chickens. This organism goes through a typical sporozoan life cycle and, with the exception of sporulation of the oocyst, all stages occur within the host's intestinal tract. Invasion of the intestinal epithelium by sporozoites and merozoites results in considerable tissue destruction and, in acute cases, severe hemorrhaging by the fifth day of infection, at which time mortality among the hosts reaches a peak.

It has been known for some time that under certain poorly defined conditions intestinal bacteria of the chicken may invade the tissues and set up local infections in the liver, spleen, and other organs. Morse (1908) frequently found "*Bacterium aerogenes*," "*B. coli*," "*B. proteus*," and "*Bacillus pyocyaneus*" in various organs of chickens which had succumbed to "white diarrhea." Hadley (1909) found bacterial invasion of the viscera in chickens infected with *Coccidium cuniculi*. This bacterial invasion was studied further by Fantham (1910), who employed coccidia-infected wild grouse. He explained that the sporozoites and merozoites act "as inoculating needles," permitting injurious bacteria to pass into the tissue of the gut, "whence, by way of the blood and lymph, they can reach other organs." Many other workers have reported similar results, which are well reviewed and discussed by Ott (1937). The latter isolated *Escherichia coli*, *Staphylococcus albus*, *Salmonella pullorum*, and an unidentified coccus from the livers of 36 out of 46 one-year-old White Leghorn hens infected with *Eimeria tenella*. Ott found little evidence of bacterial invasion in normal, *E. tenella*-free chickens. By feeding broth cultures of *E. coli* to infected hens, he was able to increase the percentage of livers infected with this bacterium. Ott concluded that there is some relationship between cecal bacteria and cecal coccidiosis.

Recently, Mann (1947) reported considerable success in stamping out cecal coccidiosis by increasing the carbohydrate and reducing the protein and roughage components in the ration. He believed that roughage "caused sufficient damage to the mucosa to pave the way for coccidial invasion." He also thought that the presence of roughage stimulated the activity of certain intestinal bacteria, which assisted coccidial invasion. Mann (1945a,b) previously found that roughage and protein stimulated proliferation of anaerobic intestinal bacteria,

particularly those resembling *Clostridium perfringens*. Therefore, Mann concluded that a particular type of cecal bacterial flora seems to be necessary for the development of *E. tenella* in chickens. Also, as pointed out by Mann (1947), the successful use of sulfonamides in alleviating the course of coccidiosis in chickens might indicate action of the drug upon associated bacteria as well as upon coccidia.

Although many workers have shown that cecal bacteria may play an active part in promoting the incidence and severity of cecal coccidiosis in chickens, no extensive study has been made of the kinds and numbers of microorganisms found in the cecum during infection with *E. tenella*. It was thought that such a study might contribute to our knowledge of the etiology of cecal coccidiosis.

TABLE 1

Composition of synthetic diet fed chicks infected with cecal coccidiosis (Cravens, 1947)

Sucrose	64%
Casein (alcohol extracted)	18
Gelatin	5
Salts IV	5
Soybean oil	5
Fish oil (2000 A, 400 D)	1
Thiamine HCl	4 mg/Kg
Riboflavin	6
Nicotinic acid	50
Ca-pantothenate	15
Pyridoxine HCl	4
Alpha tocopherol	3
Biotin	0.02
2-Methyl-1,4-naphthoquinone	0.2
Choline	0.15%
Liver L	2

METHODS

Animals. A pure line strain of *Eimeria tenella* (Railliet and Lucet), isolated by Professor C. A. Herrick of the Zoology and Veterinary Science Departments of this university, was used in infecting the chicks. The method of infection used was that of Edgar and Herrick (1944). Two-week-old hybrid Single Comb White Leghorn (female) × New Hampshire (male) chicks were employed in this investigation. They were usually off feed for from 1 to 2 hours before infection with approximately 10,000 sporulated oocysts. Wisconsin no. 45 mash (Halpin *et al.*, 1944) was fed the chicks during the pre-experimental, 2-week period. After infection the chicks were divided into two groups: one was continued on the same grain mash, and the other was placed on a nutritionally adequate synthetic ration (table 1).

Microbiological determinations. Two to four animals were picked at random just prior to infection and killed so that normal cecal samples could be obtained. The samples were prepared for bacterial study in the manner previously described for laying hens (Johansson *et al.*, 1948). In the first experiment, only four types

of cultures were prepared: carrot-liver extract ("CL") agar shake tubes (Garey et al., 1941), aerobic agar plates (Johansson et al., 1948), Difco eosin methylene blue agar plates ("EMB"), and Hajna and Perry's (1943) "SF" medium (dilution counts). In the second experiment the following determinations were made: CL agar shake tube counts, aerobic agar plate counts on Baltimore Biological Laboratory's "fluid thioglycolate" medium with 1.3 per cent agar added, plate counts in the latter medium incubated anaerobically in an atmosphere of hydrogen, EMB agar plates, and total spore counts. Spore counts were made by heat-shocking the initial 1:100 dilution (after inoculations had been made in the other media) at 80 C for from 10 to 11 minutes and inoculating tap water dilutions of this material into the following media: typtone yeast extract glucose agar (Johansson et al., 1948) plates; "thioglycolate medium" (Baltimore Biological Laboratory) plates with 1.3 per cent added agar; and shake tubes of the latter medium. Thioglycolate agar plates were incubated anaerobically in hydrogen, and the other plates and tubes were incubated aerobically. All cultures were incubated at 37 C except SF dilution counts, which were incubated at 45 C. Spore plates and tubes were incubated only 24 hours because longer incubation resulted in spreading growth of some of the aerobic sporeformers, which obscured many of the smaller colonies. CL shake cultures and SF dilution tubes were held 72 hours in the incubator, and all other cultures were incubated for 48 hours before making counts. Shake and plate cultures were run in duplicate and 5 tubes per dilution were used in making SF dilution counts. Most probable numbers of the latter were calculated from the table in *Standard Methods for the Examination of Water and Sewage* (1946).

In the first experiment on infected chicks each of the three trials was followed through to the sixth day of infection. Four chicks at a time were taken from each ration just before infection, and on the second, fourth, and sixth day of infection, for bacterial counts on the cecal pouch contents. In the second experiment cecal samples were taken from each of three or four chicks at the time of coccidial infection, and again on the third and fifth day after infection. However, each trial initiated during the second experiment was not followed through because of scheduling difficulties, i.e., the first trial might be represented by samples at 0, 3, and 5 days, while samples from the next trial might have been taken only on the fifth day of infection. Occasional samples were taken from uninfected chicks between the ages of 16 and 20 days that were maintained on both grain and synthetic rations in order to detect any changes that might occur naturally with advance in age of the chicks. All counts are expressed as the number of bacteria per gram of cecal contents; usually the samples were too small to permit dry weight determinations.

Isolation and characterization of enterococci and anaerobes were attempted during the second experiment. Enterococci were isolated from SF dilution tubes by plating out in tryptone yeast extract glucose agar containing 6.5 per cent NaCl and incubated at 37 C for 3 days. Colonies were picked from the salt agar plates and kept on slants of the agar (without NaCl) for further study. The action of these isolates on blood was determined in Difco blood agar base

containing 5 per cent bovine blood. Their ability to liquefy gelatin was determined by stab inoculation into 10 per cent Difco gelatin and incubation at 37 C.

Potential anaerobes were isolated from anaerobic thioglycolate agar plates (both spore and nonspore plates), and sometimes from CL agar shakes that evidenced excessive gassing. Isolation was accomplished in freshly steamed "thioglycolate medium" containing excess $CaCO_3$; isolates were then incubated at 37 C until adequate growth occurred. Each isolate was subsequently plated out by loop dilution in thioglycolate agar and incubated anaerobically for from 24 to 48 hours. A single, well-isolated colony was then picked into the modified Brewer's thioglycolate medium, incubated at 37 C until growth reached a maximum, and put away in the icebox for future study. Some of these cultures were anaerobic or microaerophilic, gram-positive cocci or rods which produced little or no gas in Brewer's medium. These cultures were studied further by cultivation on tryptone liver extract glucose agar slants incubated both aerobically and anaerobically; their action on litmus milk and their ability to ferment glucose, sucrose, lactose, and maltose (fermentation basal: 0.5 per cent yeast extract, 0.3 per cent tryptone, and 0.1 per cent agar, with bromcresol purple indicator added) were observed.

Nearly one-fifth (39) of the "anaerobe" isolates produced stormy fermentations in glucose- or lactose-containing media. Gram stains showed these organisms to be large, gram-positive rods; they were nonmotile and obligately anaerobic. An attempt was then made to identify these bacteria. The motility of 8- to 16-hour cultures grown in Brewer's medium was determined by microscopic examination of wet mounts. Gram stains (Kopeloff and Beerman, *Manual of Methods*, 1945) were made of 18-hour Brewer's cultures. Sporulation was induced by cultivation in Reed and Orr's (1941) proteose-peptone plating agar (adjusted to pH 8.0), incubated anaerobically in hydrogen for 48 hours. Schaeffer and Fulton's (*Manual of Methods*, 1945) spore stains were made to determine the shape and location of spores. The ability of these isolates to ferment lactose, glucose, sucrose, maltose, and sorbitol, produce H_2S, reduce nitrate, produce indole, and liquefy gelatin was determined according to the recommendations of Reed and Orr (1941). Their hemolytic action on bovine blood was tested on Difco blood agar base containing 5 per cent bovine blood. All cultures were streaked out on the egg yolk medium of McClung and Toabe (1947) and incubated anaerobically for 24 to 36 hours to observe production of lecithinase (Nagler's reaction, 1939).

Statistical analysis. A statistical analysis was made of the data from the first experiment. First, an analysis of variance between trials was made for each of the four stages of infection (0, 2, 4, and 6 days) over all cecal counts (expressed as logarithms to the base 10 carried out two places). The between-trials variation, less variation due to media, ration, and interaction of ration and media, was used to resolve significant changes in the flora with advance in infection. Then, Bartlett's chi-square test of homogeneity of variance (Snedecor, 1946) was made on the real between-trials variation of cecal counts in order to determine whether or not it was of the same magnitude at the four stages of infection.

The weighted mean of the between-trials variation was used as the variance in determining the between-days variation for each medium by another analysis of variance. Least significant differences (*LSD*) were then computed between different infection periods for those media showing significant *F* values (either at the 5 or 1 per cent level of significance). A few times missing values were estimated according to the method of Yates (Snedecor, 1946), and used in calculating the statistical average.
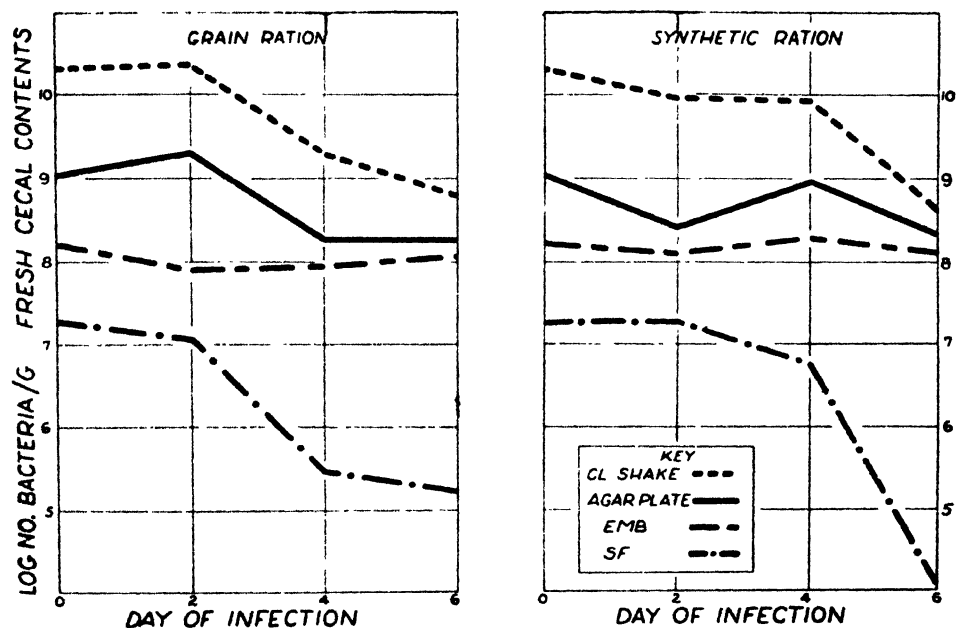
Figure 1. Changes in the cecal flora of chicks infected with *Eimeria tenella*—first experiment.

RESULTS

Experiment 1. The bacteriological results are summarized in table 2 and figure 1. The most striking changes in the cecal flora during infection with *E. tenella* appeared to exist among those groups of bacteria represented by CL and SF counts, most of which were lactic acid bacteria. After the second or fourth day of infection, very few organisms morphologically like lactobacilli could be found in CL shakes. During the latter stages of coccidial infection, CL shake tubes contained primarily coliform organisms. It is interesting to note that coliforms remained unchanged in numbers during the course of infection. Agar plate counts on cecal contents appeared to drop a little in birds on grain, while a slight change occurred in cecal plate counts of chicks on the synthetic ration. The over-all change in the cecal flora was practically the same with either ration. Lactic acid bacteria from chicks on the synthetic diet did not decrease appreciably in numbers until after the fourth day of infection, whereas in birds on the

grain mash, cecal lactics decreased after the second but before the fourth day of infection. The coliforms, as in the intestinal flora of pullets (Johansson *et al.*, 1948), appeared to be predominately *Escherichia* types.

Statistical treatment of these data revealed the difference between trials to be highly significant before coccidial infection, and on the fourth and sixth day after infection; on the second day the between-trials variation was not significant. One thing revealed by analysis of variance between trials is that the within-trials variation (the variance) gradually increased during the advance of infection (from 0.1362 to 0.6747). This indicates that variation between cecal counts increased during the course of cecal coccidiosis. Bartlett's chi-square test of the homogeneity of between-trials mean squares resulted in a slightly over 5 per cent level of probability, indicating that these mean squares were not

TABLE 2

Cecal counts of chicks infected with Eimeria tenella—Experiment 1

MEDIUM	DAYS INFECTED						
	0	2		4		6	
	Grain	Grain	Synthetic	Grain	Synthetic	Grain	Synthetic
CL*	19,884.0	21,822.5	9,108.0	1,920.6	8,509.3	647.7	426.0
	(9)	(4)	(6)	(6)	(6)	(6)	(6)
Agar plate*	1,076.9	2,031.3	254.7	187.8	922.3	187.5	216.2
	(10)	(4)	(6)	(6)	(6)	(6)	(6)
EMB*	130.7	79.8	125.5	90.6	212.3	111.2	134.7
	(10)	(4)	(6)	(6)	(6)	(6)	(6)
SF†	1,953.8	1,236.5	1,965.0	30.7	613.2	16.5	1.0
	(10)	(4)	(6)	(6)	(6)	(6)	(6)

Numbers in parentheses indicate the number of samples from which the average was obtained.

* Counts expressed $\times 10^6$.

† Counts expressed $\times 10^4$.

homogeneous. Actually, one mean square (at 2 days after infection) was out of line and, since a better variance was not available, the weighted mean of the between-trials mean squares was employed in determining the variation between days (Torrie, 1948). Perusal of table 3 indicates the following: (1) CL and agar plate counts changed significantly between days of infection in the ceca of chicks on grain, but not in chicks on the synthetic ration; (2) no significant change in coliform counts occurred in cecal coliforms of chicks on either ration; and (3) the decrease in enterococcus counts during infection was highly significant for both rations. Least significant difference calculations revealed that the drop in CL counts of chicks ingesting grain was not significant until the fourth day of infection ($LSD_{.05}$ log 1.37). Similarily, cecal agar plate counts of grain-fed chicks did not change significantly until the sixth day of infection ($LSD_{.05}$ log 1.34). A highly significant drop in numbers of cecal enterococci was found to occur on the fourth day of infection in chicks ingesting grain, and on the sixth day of infection in chicks on the purified diet ($LSD_{.01}$ log 1.79).

Experiment 2. The results of this study are compiled in table 4 and figure 2. As in the first experiment, the CL shake counts decreased more in the ceca of coccidia-infected chicks on grain than in those on the synthetic diet. Aerobic and anaerobic thioglycolate agar plate counts were surprisingly similar and changed most in birds on grain; in chicks on the synthetic ration these two plate counts appeared to remain fairly constant during infection. Since it was discovered that coliforms made up the majority of the colonies growing on these plates, it is not surprising that anaerobic and aerobic plate counts were similar

TABLE 3

Analysis of variance for between-days' differences in cecal counts

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES	MEAN SQUARE	F VALUE
1. Carrot-liver agar shakes				
Between days, grain....	3	17.2741	5.7580	3.405*
Between days, synthetic.............	3	6.9801	2.3267	1.376
Between trials......................	52	87.9361	1.6911	
2. Agar plates				
Between days, grain...........	3	14.2101	4.7367	2.801*
Between days, synthetic...	3	3.9831	1.3277	0.785
Between trials......................	52	87.9361	1.6911	
3. Coliform counts				
Between days, grain................	3	6.8276	2.2759	1.346
Between days, synthetic............	3	0.6098	0.2033	0.120
Between trials......................	52	87.9361	1.6911	
4. Enterococcus counts				
Between days, grain................	3	54.0650	18.0217	10.657†
Between days, synthetic............	3	61.6060	20.5353	12.143†
Between trials......................	52	87.9361	1.6911	

* *F* significant at the 5 per cent point.
† *F* significant at the 1 per cent point.

in magnitude. Especially in chicks on the synthetic diet, it may be noted that coliforms made up by far the majority of the cecal flora after 5 days' infection with *E. tenella.* It is believed that both CL shake and plate counts were merely coliform counts at this late stage of infection. Lactobacilli were rarely encountered in CL shakes after the third day of infection. Anaerobic spore counts[4] decreased during infection, while aerobic spore counts of cecal contents remained essentially unchanged. Thioglycolate agar shake tube counts of spores markedly

[4] It was found that many of the "aerobic" sporeformers grew well in an atmosphere of 100 per cent hydrogen; hence the anaerobic spore count includes a fair number of aerobes which obscure the true decrease in numbers of anaerobic spores.

TABLE 4

Cecal counts of chicks infected with Eimeria tenella—Experiment 2

MEDIUM	DAYS INFECTED				
	0	3		5	
	Grain	Grain	Synthetic	Grain	Synthetic
CL*	6,942.0 (10)	11.076.0 (8)	4,429.4 (8)	745.3 (8)	930.2 (8)
Thioglycolate plates—anaerobic*	1,826.8 (10)	1,335.4 (8)	3,836.3 (8)	169.1 (8)	789.6 (8)
Thioglycolate plates—aerobic*	1,889.3 (10)	1,804.1 (8)	3,115.2 (8)	140.1 (8)	739.1 (8)
EMB*.	359.6 (10)	42.4 (8)	2,630.8 (8)	73.3 (8)	760.1 (8)
Spore shakes†	171.0 (7)	515.0 (8)	553 0 (6)	79.4 (5)	25.9 (6)
Spore plates—anaerobic†	1,484.0 (10)	215 6 (8)	529.0 (8)	19.0 (6)	230 7 (6)
Spore plates—aerobic†	405.0 (10)	627 4 (8)	210.8 (8)	717.1 (6)	948.5 (6)

Numbers in parentheses indicate the number of samples from which the average was obtained.

* Counts expressed × 10^6.

† Counts expressed × 10^3.
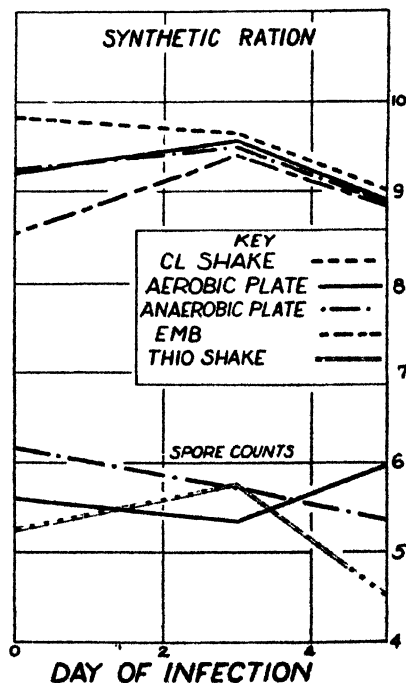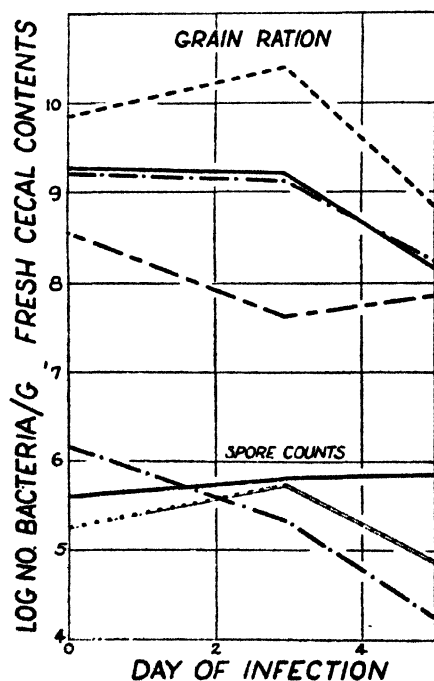
Figure 2. Changes in the cecal flora of chicks infected with *Eimeria tenella*—second experiment.

decreased after the third day. There appeared to be little difference in cecal flora changes between birds on the two rations, but chicks on the synthetic diet possessed a slightly richer cecal coliform flora throughout coccidial infection.

The cecal flora of normal, uninfected chicks of the same age as those that were infected was found to remain the same; hence these changes in the flora which were found to occur in both experiments must not be due to age but to some other factor, presumably, the coccidial infection.

Large, cream-colored lenticular colonies, which are typical of *Clostridium perfringens*, usually were noticed in spore plate counts made before infection on cecal contents diluted as high as 10^{-4} or 10^{-5}; nonspore anaerobic plates of cecal contents rarely produced such colonies beyond 10^{-5} dilution prior to infection. However, on the fifth day of infection, *C. perfringens*-like colonies often were found in abundance on anaerobic plates of the unheated material up to the 10^{-8} dilution, but were rarely seen in the lowest dilution (10^{-2}) of the anaerobic spore plates. These colonies were easily recognized, and when transferred to litmus milk containing reduced iron, always gave a stormy fermentation. Their presence in thioglycolate agar shake tubes was unmistakable because they blew the agar to bits and pushed the cotton plugs completely out of the tubes within 18 hours at 37 C. Thus, it seems that changes in the ceca during infection with coccidia resulted in favorable conditions for growth of the gas-gangrene organism as evidenced by germination of *C. perfringens* spores and their extensive proliferation.

One hundred seventy-six isolations were made from CL agar shake cultures and anaerobic plates (both of heat-shocked and non-heat-shocked preparations). Of this total, 39 were identified as *C. perfringens* and the rest were found to be chiefly coliform bacteria, cocci, and lactobacilli, or unidentified microaerophilic or anaerobic gram-positive rods. All cultures identified as *C. perfringens* fermented glucose, lactose, maltose, and sucrose, produced a stormy fermentation in litmus milk, reduced nitrate to nitrite, did not produce indole, produced H_2S, and gave a positive lecithinase reaction. Only one such culture did not liquefy gelatin, and only four did not hemolyze bovine blood (the others produced a weak to strong alpha hemolysis). Spores of a few of the cultures were observed and found to be oval, excentric to subterminal in location; the sporangia were slightly swollen. Sporulation of the isolates was induced with great difficulty, and only a few spores were seen in any culture. The cultures were nonmotile, gram-positive, and were fairly large regular rods. Thus, the morphological, cultural, and biochemical studies of these sporeforming anaerobes seems to agree with the description of *C. perfringens* given in *Bergey's Manual of Determinative Bacteriology* (1948).

Eight cultures of enterococci were isolated from SF dilution cultures of eight different coccidia-free chicks, varying in age from 7 to 30 days. They were all large, irregularly-shaped, gram-positive cocci, able to grow in a medium containing 6.5 per cent NaCl, to produce either alpha or gamma hemolysis of bovine blood, and unable to liquefy gelatin. From this preliminary study of a few enterococcus isolates from young chickens, it appears that they are *Streptococcus faecalis*.

Seven gram-positive, anaerobic or microaerophilic nonsporeforming rods found among the 176 isolates appeared to be lactobacilli. Six of them fermented sucrose, maltose, lactose, and glucose, while one fermented sucrose, maltose, and glucose with the production of acid but no gas. Growth, if any, on aerobic slants was scant, whereas stab cultures grew well. None strongly coagulated or reduced litmus milk, but all produced acid in this menstruum. No attempt was made to classify these isolates as to species.

A few of the microaerophilic coccus isolates were inoculated into tubes of SF medium and incubated at 45 C; no growth appeared during 1 week of incubation. This indicates that some of the coccus isolates were not enterococci. They were found to ferment sucrose, maltose, lactose, and glucose with the production of acid but no gas. Further study of these coccus isolates was not made.

<div align="center">DISCUSSION</div>

There have long been indications that the etiology of cecal coccidiosis is complex, and that destruction of intestinal epithelium with resultant hemorrhage by the protozoan parasite, *Eimeria tenella*, is but one aspect of the disease. Bacteria belonging to the coliform, coccus, and sporeforming anaerobe groups have been frequently isolated from the liver, spleen, heart, and blood of chickens which have succumbed to coccidiosis infection (Morse, 1908; Hadley, 1909; Fantham, 1910; Ott, 1937). No doubt secondary infection is faciliated as a result of weakened cecal walls and a general emaciation of the animal. Mann (1947) goes further and claims that "coccidiosis in chicks is a secondary complaint, the primary aetiological agents being bacteria which proliferate to excess in the presence of roughage or protein." In addition, it is possible that toxic products of bacteria may penetrate the infected epithelium to confuse the picture even more.

Mann (1945a, b) studied "six-day disease" of chicks, which he found to be caused by invasion by various fecal bacteria, chiefly *Clostridium welchii*, enterococci, and coliforms, through the intestinal wall. His description of the appearance of the cecal pouches of birds afflicted with this infection is similar to that of cecal coccidiosis: occlusion of the ceca by a hard core with hemorrhage in varying degrees. He was able to control this disease by using a ration high in natural carbohydrate, and low in animal protein, roughage, and residual oils capable of destroying vitamin A. McGaughey (1944) reported similar results in a bacteriological study of "six-day disease" of chicks. He was able to reproduce the disease by feeding cultures of *C. welchii* to day-old chicks. McGaughey suggested that the cause of death, which is fulminating, might be a *C. welchii* intoxication. Mann (1947) believes the bacteriology of cecal coccidiosis to be similar to that of "six-day disease," and that it too could be controlled by a ration like the one employed to subdue morbidity due to "six-day disease."

The results of the first experiment show that coliform organisms, primarily *Escherichia* species, do not decrease in numbers in the ceca during the course of coccidiosis, whereas lactic-acid-producing bacteria all but disappear. The second experiment agreed with the first and, in addition, indicated that condi-

tions in the ceca of infected chicks were modified so as to favor proliferation of sporeforming anaerobes, especially *C. perfringens*. Marsh and Tunnicliff (1944) found many coccidia-infected sheep to develop enterotoxemias caused by *C. perfringens* (type D). Therefore, the results obtained in this study strengthen the belief that *C. perfringens* may be a secondary invader in chicks infected with coccidia, and that the activity of *E. tenella* makes their growth possible.

There is considerable evidence in the literature that injury to the intestinal tract of an animal may result in visceral invasion by *C. perfringens*. Williams (1927) pointed out that, when the small bowel of man becomes paralyzed or obstructed, conditions become ideal for growth of *Bacillus welchii*. Stabins and Kennedy (1929) artificially obstructed the jejunum of dogs and found an abrupt increase in numbers of *C. welchii* within the obstructed segment. *C. welchii* was found to invade the blood stream in five human cases in which the primary lesion was in the intestinal tract (Cruickshank and Davidson, 1944). The latter state: "Injury and disease of the alimentary canal, which normally harbours organisms of the gas gangrene type. . . may allow the entry of these organisms into the blood stream." Since cecal coccidiosis of the chicken results in occlusion of the cecum together with injury to the intestinal mucosa and epithelium, a favorable environment is established for the growth of *C. perfringens*. None of the *C. perfringens* isolates were typed in the present study, but Taylor and Gordon (1940) found only type A among the 88 cultures isolated from 12 of 13 chickens examined.

The coliform organisms may also be involved in the etiology of cecal coccidiosis. This study has shown that they are present in large numbers throughout the course of coccidiosis infection. In a previous investigation (Johansson *et al.*, 1948) it was found that the cecal contents of mature hens had a coliform count considerably lower than that found in the ceca of young chickens. This might possibly be a factor related to the reduced severity of cecal coccidiosis which is known to occur with advance in the age of chickens (Herrick *et al.*, 1936).

Although no outstanding difference was noted between the cecal flora of chicks on the two rations employed in this work, Herrick (1947) has found the grain mash to favor an appreciably higher incidence, severity, and mortality in the chicks infected with *E. tenella* than did the purified ration.

SUMMARY AND CONCLUSIONS

The cecal flora of 2-week-old chicks on a grain mash or on a synthetic diet changed during the course of infection with *Eimeria tenella*. Numbers of lactobacilli and enterococci were reduced considerably by the fourth or fifth day of infection. Coliform organisms were found to remain unchanged in numbers in the ceca during cecal coccidiosis. The growth of anaerobes resembling *Clostridium perfringens* was stimulated by coccidial infection of the cecum.

A statistical analysis was made of the enumerative data from one of these experiments and revealed that some of the changes which occurred in the cecal flora of chicks maintained on two different rations during the course of coccidial infection were statistically significant.

It is believed that *C. perfringens* and *Escherichia coli* complicate the etiology of cecal coccidiosis of the domestic chicken.

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A COMPARATIVE STUDY OF THE IN VITRO BACTERIOSTATIC ACTION OF SOME SIMPLE DERIVATIVES OF FURAN, THIOPHENE, AND PYRROLE

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A study of the *in vitro* bacteriostatic action of simple furan derivatives has demonstrated the value of the nitro group in the molecule for effective bacteriostasis of both gram-positive and gram-negative organisms, and the inactivity of analogous nonnitrated compounds (Dodd and Stillman, 1944). The proved function of the nitro group in the furan series created interest in its effect when introduced into other five-membered heterocyclic ring systems having a single hetero-atom. Thiophene and pyrrole, the sulfur and nitrogen analogues of furan, which have physical and chemical characteristics that resemble furan in many respects, were chosen for a comparative study of the effect of a nitro group on the bacteriostatic action of such compounds.

A series of simple thiophene and pyrrole derivatives both nitrated and nonnitrated was, therefore, prepared and tested for *in vitro* bacteriostatic action and the results were compared with corresponding furan derivatives.

Certain furan derivatives having a substituent in the 2-position containing an aldehyde or ketone carbonyl group and nitrated in the 5-position are highly efficient bacteriostatic agents. The compounds 5-nitro-2-furaldehyde and 5-nitro-2-acetofurone are excellent examples of active compounds of this type. The former and its semicarbazone derivative are two of the most active members of the furan series. The 2-acetyl derivatives of thiophene and pyrrole were prepared and compared with the active 2-acetyl furan derivatives.

Nonnitrated and nitrated compounds are included for comparison. The effect of introducing a nitro group is clearly brought out. The data on bacteriostatic activity of 2-acetofurone semicarbazone and 5-nitro-2-acetofurone semicarbazone have not been published.

In the thiophene and pyrrole series the effect of a nitro group in the 4-position on *in vitro* bacteriostatic action was observed. The inability to prepare the corresponding simple furan derivative precluded any observation of the effect of a 4-nitro substituent in this series.

The following tables show the *in vitro* bacteriostatic action of the 16 compounds comprising this study. The testing procedure has been described (Dodd and Stillman, 1944)

From table 1 it is apparent that the nonnitrated furan derivatives are not bacteriostatic but the introduction of a nitro group has conferred bacteriostatic properties upon 2-acetofurone and upon its semicarbazone, although the action

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of the latter compound is of a lower order than the ketone itself. It is believed that the insolubility of the semicarbazone prevents manifestation of any activity

TABLE 1

Bacteriostatic action of furan derivatives

(Minimum bacteriostatic concentration in mg per cent after 24 and 96 hours' incubation)

COMPOUNDS	S. AUREUS		S. HEMOLYTICUS		D. PNEUMONIAE I		E. TYPHOSA		E. COLI		P. PYOCYANEA	
	24	96	24	96	24	96	24	96	24	96	24	96
I. 2-Aceto-furone	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
II. 2-Aceto-furone semi-carba-zone.	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
III. 5-Nitro-2-aceto-furone.	0.5	1.66	0.5	1.66	0.2	1.25	0.5	1.0	1.0	1.0	20.0	20.0
IV. 5-Nitro-2-aceto-furone semi-carba-zone.	1.25	2.5	2.5	3.33	>3.33	>3.33	1.25	2.5	3.33	>3.33	>3.33	>3.33

TABLE 2

Bacteriostatic action of thiophene derivatives

(Minimum bacteriostatic concentration in mg per cent after 24 and 96 hours' incubation)

COMPOUNDS	S. AUREUS		S. HEMOLYTICUS		D. PNEUMONIAE I		E. TYPHOSA		E. COLI		P. PYOCYANEA	
	24	96	24	96	24	96	24	96	24	96	24	96
V. 2-Acetothi-enone.	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
VI. 2-Acetothi-enone semi-carbazone .	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
VII. 5-Nitro-2-acetothi-enone	2.5	>3.33	>1.0	>2.5	1.0	1.25	1.25	2.5	5.0	5.0	>20.0	>20.0
VIII. 5-Nitro-2-acetothi-enone semi-carbazone..	>2.5*	>2.5	>2.5	>2.5	2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
IX. 4-Nitro-2-acetothi-enone......	>50†	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
X. 4-Nitro-2-acetothi-enone semi-carbazone..	>5‡	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5

Maximum solubility in broth—*2.5, †50, ‡5 mg per cent.

against *Diplococcus pneumoniae* I or *Pseudomonas pyocyanea* (*Pseudomonas aeruginosa*).

These findings substantiate the results previously reported in which only 3 nonnitrated furan compounds out of the 17 tested showed very slight bacteriostatic action, whereas 24 of the 25 nitrated compounds demonstrated activity at high dilutions.   The lack of activity of the one compound in the latter series was ascribed to its low solubility in the test broth (1.5 mg per cent).

The thiophene derivatives presented in table 2 show in part the trend noted in the furan series.   The nonnitrated derivatives are inactive at high concentrations, but the introduction of a nitro group in the 5-position has conferred bacteriostatic action upon 2-acetothienone which is of an order comparable to nitrofurans.   The semicarbazone of 5-nitro-2-acetothienone shows bacteriostatic

TABLE 3

Bacteriostatic action of pyrrole derivatives

(Minimum bacteriostatic concentration in mg per cent after 24 and 96 hours' incubation)

COMPOUNDS	S. AUREUS		S. HEMOLYTICUS		D. PNEUMONIAE I		E. TYPHOSA		E. COLI		P. PYOCYANEA	
	24	96	24	96	24	96	24	96	24	96	24	96
XI. 2-Acetyl pyrrole....	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
XII. 2-Acetyl pyrrole semicarbazone.	>100	>100	40	40	>100	>100	40	40	>100	>100	>100	>100
XIII. 5-Nitro - 2 - acetyl pyrrole......	100	>100	20	100	>100	>100	40	40	40	40	100	>100

XIV. 5-Nitro - 2 - acetyl pyrrole semicarbazone	Solubility less than 5 mg %	No bacteriostatic action in saturated solution
XV. 4-Nitro - 2 - acetyl pyrrole	Solubility less than 40 mg %	No bacteriostatic action in saturated solution
XVI. 4-Nitro - 2 - acetyl pyrrole semicarbazone	Solubility less than 4 mg %	No bacteriostatic action in saturated solution

action only against *D. pneumoniae* I at the limit of its solubility (2.5 mg per cent) in contrast to the semicarbazone of 5-nitro-2-acetofurone.   It seems apparent from this that a nitro group may confer bacteriostatic action on thiophene compounds, but it is probably not so effective as it was with nitrofurans.

The isomeric 4-nitro-2-acetothienone is to all intents inactive, as is its semicarbazone.   In the case of the semicarbazone, solubility is a limiting factor, but the ketone itself has a solubility of 50 mg per cent, which is comparable to the active 5-nitro derivative.   This would lead to the conclusion that the introduction of the nitro group into the 4-position of the thiophene ring does not confer the bacteriostatic effect noted when the substitution takes place in the 5-position.

Table 3 presents the *in vitro* bacteriostatic action of the pyrrole derivatives. In this series one of the nonnitrated compounds, 2-acetyl pyrrole semicarbazone,

showed bacteriostatic action against two of the six organisms tested in contrast to the findings in the furan and thiophene series. Also, the presence of a nitro group in the 5-position not only failed to maintain the activity found in this compound but conferred only slight activity on the other derivative tested, 2-acetyl pyrrole. Again the 4-nitro compounds were inactive.

From the data presented here on the *in vitro* bacteriostatic activity conferred upon a limited series of simple derivatives of furan, thiophene, and pyrrole by the introduction of a nitro group, this order of activity is shown: furan > thiophene > pyrrole, with the pyrrole derivatives practically devoid of activity.

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THE CULTURE AND PHYSIOLOGY OF A THERMOPHILIC CELLULOSE-FERMENTING BACTERIUM[1]

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Many attempts to isolate thermophilic cellulose-fermenting bacteria have failed to yield cultures of undoubted purity. Because of the lack of pure cultures conflicting and erroneous ideas regarding the cultural requirements and metabolic characteristics of these organisms have arisen. Recently, with the procedure developed by Hungate (1944, 1947), pure cultures have been obtained and studies made on the metabolism of the cellulose-fermenting thermophiles.

ISOLATION OF PURE CULTURES

Enrichment cultures from soil or manure were made at 55 C in stoppered bottles containing medium of the following percentage composition: NaCl, 0.6; $(NH_4)_2SO_4$, 0.1; KH_2PO_4, 0.05; K_2HPO_4, 0.05; $MgSO_4$, 0.01; $CaCl$, 0.01; and cellulose, 0.5. The cellulose had been prepared from absorbent cotton according to the procedure of Farr and Eckerson (1934). A 5 per cent suspension was ground with distilled water in a pebble mill for 72 hours. This gave a thick slurry of finely divided cellulose, which was used in all the experiments.

Cellulose fermentation in the enrichment cultures occurred in a few days as evidenced by the formation of gas bubbles and a tendency for the cellulose to become mucilaginous. When fermentation was active approximately 0.1 ml of the sediment containing the cellulose was transferred to a test tube of sterile enrichment medium plus 0.1 per cent yeast extract. This culture served as a starting point for attempts at isolation of pure cultures.

Serial shake dilutions of these cultures were made in cellulose agar of similar composition. Cellulose digestion was observed in all these agar dilution series. It was manifested either by a general clearing of the opaque white cellulose or by the formation of a clear zone around a discrete central colony. In the first instance, transfers from the cleared agar to further cellulose agar series finally gave isolated colonies surrounded by clear zones. These isolated colonies were subcultured to further similar series except that filtered glucose was substituted for the cellulose. The resulting colonies were again returned to cellulose. No growth resulted. This resembled the results previously reported by other investigators (Viljoen, Fred, and Peterson, 1926), namely, failure to digest cellulose after growth on glucose. However, further study showed that these colonies in glucose were actually heat-resistant contaminants present in the agar. By auto-

claving for 30 minutes at 20 pounds pressure these contaminants were killed. But in this adequately sterilized medium to which filtered glucose was added no growth whatsoever took place. Failure to obtain growth was not due to damage to the medium by the sterilization process since the cellulose medium when similarly sterilized supported excellent development. These results suggested that the organisms would not grow on glucose. It seemed highly desirable, however, to obtain dilution series in a sugar medium, so cellobiose was tested. Dilution into a cellobiose agar series showed colony development as good as that obtained in cellulose media. A second subculture in cellobiose and then a return to cellulose gave active cellulose digestion.

The use of cellobiose agar was the key to a pure culture, because it permitted repeated transfers from isolated colonies in high dilutions in a transparent solid medium in which any contaminants could be detected. The criteria for culture purity that have been followed are: (1) The strain must arise from a single, well-isolated colony in cellulose agar. (2) Upon dilution into cellobiose agar it must yield only one colony type. (3) A single isolated colony from a high dilution of the cellobiose agar must be transferred to a second cellobiose agar dilution series with similar results. (4) All the colonies in the highest dilution showing growth in the second cellobiose agar series must be macroscopically similar and yield only cellulose-digesting colonies when transferred into cellulose agar.

Fourteen strains, all similar to one another, were cultured from the initial enrichments, of which two, cultures 157 and 651, have been subjected to the foregoing purity tests. The remaining 12 strains were not carried beyond the first few transfers because they appeared identical with the two isolates. Culture 157 has been used for most of the physiological work.

PHYSIOLOGY

Quantitative fermentation studies. The products of cellulose fermentation have been determined for the pure cultures. The culture technique was essentially the same as that described by Hungate (1944) using all-glass culture flasks with inlet and outlet tubes that were sealed in a flame after inoculation. The medium was prepared with a phosphate-buffered inorganic salt base. The amount of cellulose was determined from a control flask in each series. Yeast extract was omitted since it was found that sufficient growth factors were present in a 1 per cent inoculum from a 0.05 per cent yeast extract cellulose culture. Substitution of sodium sulfide for sodium thioglycolate, the usual reducing agent, further reduced the amount of extra carbon. Anaerobiosis was obtained by evacuating the flasks after inoculation. The inoculated control flask was refrigerated.

The time required for the disappearance of all the cellulose in the experimental flasks at 55 C varied from 5 to 40 days, according to its concentration and the inoculum.

Experiment 1. The first series of quantitative cultures contained 74.4 mg of cellulose in 100 ml of medium (0.0744 per cent cellulose). The cellulose com-

pletely disappeared in 5 days at 55 C, at which time no trace of reducing sugars could be detected. The amounts of fermentation products found upon analysis of duplicate flasks are shown in table 1. The values in the two flasks agree, except in the case of carbon dioxide, in which some error in flask 1 may have been due to a lack of equilibrium in the ascarite absorption train before the first analysis was started. All values are the average of duplicate analyses which generally checked within 5 per cent. No acetylmethylcarbinol, 2,3-butylene glycol, or methane were found. No alcohols or fatty acids higher than ethanol or acetic acid were present.

The total amount of carbon found in the fermentation products is only about 70 per cent of the initial carbon of the cellulose. This amount, peculiarly, is approximately the recovery obtained by Khouvine (1923) in her analyses; by

TABLE 1

Fermentation balance, culture 157, experiment 1

FERMENTATION PRODUCTS	EXPERIMENTAL FLASK 1			EXPERIMENTAL FLASK 2		
	Mg	mM	Mg atom carbon	Mg	mM	Mg atom carbon
	Initial cellulose					
	74.4		2.76	74.4		2.76
Carbon dioxide............ ..	27.2	0.62	0.62	13.2	0.30	0.30
Hydrogen....	0.84	0.42	—	1.14	0.57	—
Ethyl alcohol...	9.67	0.21	0.42	9.42	0.20	0.40
Formic acid.......	10.4	0.23	0.23	9.70	0.21	0.21
Acetic acid....	16.2	0.27	0.54	12.5	0.21	0.42
Lactic acid....	9.84	0.11	0.33	10.7	0.12	0.36
Succinic acid.	0.39	0.003	0.012	0.66	0.005	0.02
Residue (cells)............ ..	1.6	—	—	2.0	—	—
Total			2.152			1.71
Carbon recovery.....			78%			62%

Hungate (1944) in the fermentation balances of *Clostridium cellobioparus*; and by Imsenecki (1939) in the studies on his thermophilic cellulose-fermenting organism. In the present experiments the recovered fermentation products possess a degree of oxidation somewhat higher than that of the substrate. This indicates that the undetermined carbon should be found in compounds slightly more reduced than carbohydrates. In a later experiment a small amount of glycerol was found. This could account for some of the missing carbon in table 1, but scarcely all of it. The discrepancy is considerably larger than the error in analysis, indicating that one or more important fermentation products have not been recognized.

An important result of this experiment was the demonstration that reducing materials are not formed during fermentation. In additional experiments using 0.1 and 0.15 grams of cellulose per 100 ml it has always been found that no reducing sugar accumulated.

Experiment 2. A fermentation of 1.484 g of cellulose in 500 ml (0.298 per cent) was conducted. All the cellulose had disappeared after 7 days' incubation. Essentially the same fermentation products were found and they occurred in approximately the same proportion as in the first experiment (table 2). In addition to the fermentation products there was found also to be a significant accumulation of copper-reducing materials. In all similar cultures containing more than 0.15 per cent cellulose, reducing materials accumulated in the medium unless the culture was terminated at or before the period of most active fermentation.

In several experiments it has been found that the amount of reducing materials increases after growth has stopped. This suggests that hydrolysis can occur independently of fermentation. Fermentation ceases because of toxic end prod-

TABLE 2

Fermentation balance, culture 157, experiment 2

FERMENTATION PRODUCTS	EXPERIMENTAL FLASK 1			EXPERIMENTAL FLASK 2		
	Mg	mM	Mg atom carbon	Mg	mM	Mg atom carbon
	Initial cellulose					
	1,484		54.9	1,484		54.9
Carbon dioxide................	160.8	3.66	3.66	144.7	3.29	3.29
Hydrogen...................	7.06	3.50	—	7.10	3.55	—
Ethyl alcohol..............	68.6	1.49	2.98	64.6	1.40	2.80
Formic acid................	36.5	0.79	0.79	38.1	0.83	0.83
Acetic acid.................	136.8	2.28	4.56	134.4	2.24	4.48
Lactic acid.................	82.6	0.92	2.76	79.0	0.88	2.64
Succinic acid......	0			0		
Glucose....................	480.0	2.66	·16.0	480.0	2.66	16.0
Residue....................	11.3			7.0		
Total...................			30.75			30.04
Carbon recovery............			56%			55%

ucts, but hydrolysis continues. The normally fermented sugars then accumulate.

The carbon recovery in experiment 2 was less than that in the previous experiment, assuming all the copper reduction to be due to glucose. This lack of carbon in an experiment in which digestion occurs without accompanying fermentation suggests either that a nonreducing intermediate in the hydrolysis of cellulose had accumulated along with glucose or that the copper reduction was not due to glucose alone. Particularly cellobiose should be considered as a hydrolysis product. No evidence of cellobiose or of hydrolysis intermediates was obtained, however. Acid hydrolysis gave no increase in reduction and no hot-water-soluble osazone could be demonstrated. A hot-water-insoluble osazone which formed was identified as glucosazone by its melting point and microscopic appearance. These results suggested that glucose was the chief product of the cellulose hydrolysis.

The formation of glucose from cellulose by culture 157 coupled with its failure to ferment glucose constitutes an anomalous situation in which a hydrolytic product is formed but apparently is not utilized. Further experiments were performed in an at empt to explain this seeming discrepancy.

The nature of the hydrolytic products. Neutralized culture fluid from experiment 2 was evaporated under reduced pressure and the sugar separated from the salts by extraction with alcohol. The reducing values of the sugar and of a glucose control were not changed by heating on a steam bath for 2 hours with 5 per cent sulfuric acid. A cellobiose control showed a 40.4 per cent increase in copper reduction under the same conditions. The amount of carbon dioxide produced by yeast fermentation of the unknown sugar before and after acid hydrolysis was measured manometrically. The carbon dioxide production from the culture sugar was equivalent to and at the same rate as that from an amount of glucose with the same copper-reducing capacity. Cellobiose was not fermented by the yeast. In another culture containing initially 5 per cent cellulose a sufficient concentration (2.8 per cent) of sugar was formed to permit studying it without first extracting. The increase in copper reduction upon acid hydrolysis was 1.6 per cent for the unknown, whereas a cellobiose control gave a 40 per cent increase. Yeast fermentability was measured manometrically at 30 C. The average total gas volumes per unit of copper-reducing capacity were: glucose control of equal copper-reducing capacity, 193.2 μl; unknown sugar, 196.0 μl; unknown after acid hydrolysis, 2.04 μl. Cellobiose was not fermented. An osazone prepared from the unknown yielded a copious precipitate while hot. No cellobiosazone crystals were formed when the hot filtrate was cooled. The hot-water-insoluble precipitate was collected, washed with boiling water, and recrystallized from pyridine. The resulting crystals were microscopically similar to those of glucosazone prepared in parallel. Both decomposed at the same temperature, 207 C. This showed that, also in a culture solution which was neither evaporated nor extracted, glucose was the principal product of hydrolysis of the cellulose.

In view of the preceding evidence that glucose is actually formed by strain 157 it seemed desirable to study further the possibility of its utilization. In particular it appeared profitable to study the effect of glucose in the presence of other utilizable substrates. It had been noted during the isolation procedures that in glucose agar containing traces of cellulose the colony size was small and was proportional to the amount of cellulose digested, whereas substitution of cellobiose for glucose yielded large colonies before cellulose digestion was apparent. This suggested that cellobiose was used but glucose was not.

These observations were confirmed by quantitative experiments. Fluid media were prepared with the following carbohydrate compositions, respectively: (1) 0.1 per cent glucose, (2) 0.05 per cent glucose plus 0.05 per cent cellulose, (3) 0.05 per cent glucose plus 0.05 per cent cellobiose, (4) no carbohydrates. These were inoculated with strain 157 and copper reduction values determined on 1 ml of each culture before incubation and again after 24 hours, at which time the cellulose had disappeared. An adaptation of the Bertrand method to the determination of small amounts of sugar was used. Any growth was detected

by the production of turbidity. After fermentation, 15 ml of each culture were evaporated and osazones were formed. These were centrifuged out and the relative amounts estimated by visual comparison. The results of this experiment (table 3) show conclusively that glucose was not attacked by strain 157 even in the presence of cellobiose and cellulose, which were completely utilized.

This failure to use glucose was not due to the formation of toxic materials during heating, as Stanier (1942) had found in the case of cultures of *Cytophaga*, since all glucose used in these studies was sterilized by filtration and added to the sterile culture medium, which was not further heated.

Another possibility was investigated, namely, that the "glucose" produced in the cultures differed in some subtle fashion from the reagent glucose provided in the foregoing experiment. This was examined by the following procedure: Fluid from the 5 per cent cellulose culture, which contained 2.8 per cent "glucose" formed by hydrolysis of the cellulose, was extracted with ether to remove the

TABLE 3

Glucose fermentability

CARBOHYDRATE	GROWTH	COPPER REDUCTION VALUES		GLUCOSAZONE
		Initial	Final	
%		mg/ml	mg/ml	
Glucose 0.1........ .	None	1.41	1.41	++
Glucose 0.05 ⎫ .... Cellulose 0.05⎭....	Good	0.68	0.65	+
Glucose 0.05 ⎫ ... . Cellobiose 0.05⎭.....	Good	1.41	0.63	+
None.......................	None	0.14	0.16	None

fermentation acids which might be inhibitory to the growth of 157. It was then sterilized by filtration. The sterile solution was added to a fluid medium containing 0.01 per cent cellulose to give approximately a 0.06 per cent "glucose" concentration and a copper reduction value of 1.2 mg per ml. This was inoculated with a fluid culture of 157 and incubated at 55 C. Growth was evidenced by disappearance of the trace of cellulose within 24 hours and the ensuing turbidity of the medium. Copper reduction values determined on duplicate tubes after 5 days' incubation were 1.20 and 1.25 mg, respectively, per ml of culture, showing that there was no utilization of this "glucose" produced by the bacteria though the "glucose" was present in the same culture in which cellulose was readily fermented.

In order to eliminate the possibility in the foregoing experiment that the sugar had been altered by the procedures used to eliminate fermentation acids, the problem was approached in another manner. A culture containing 0.5 per cent cellulose was started. After complete disappearance of the cellulose in 23

days, two different dilutions of the culture were made with fresh sterile car-bohydrate-free medium: (1) one part culture fluid was diluted with 4 parts medium, and (2) two parts culture were diluted with 3 parts medium. Aliquots for analysis were removed from each dilution and the remainder was then in-cubated anaerobically at 55 C for 14 days, at which time samples were again removed. Slight growth occurred during the initial 24 hours of incubation as evidenced by turbidity in the flasks. This was possibly due to the yeast extract or to traces of cellobiose present. Copper reduction values were determined directly on the initial and final samples and also after acid hydrolysis (table 4). There was no significant change in copper reduction values during incubation of the diluted cultures. There was a small increase in copper reduction value upon acid hydrolysis both before and after incubation. It was of the same magnitude, however, in both cases and may be interpreted as being due to the presence of hydrolyzable material other than cellobiose. Inoculation of the

TABLE 4

Fermentability of accumulated sugar

DILUTION	COPPER REDUCTION VALUES	
	Culture	Hydrolyzed culture
	mg/ml	*mg/ml*
5 ×		
Initial....	1.36	1.48
After incubation	1.40	1.58
2.5 ×		
Initial	2 54	2.85
After incubation.....	2.58	2.92

diluted cultures into cellulose media showed viable organisms of strain 157 present without contamination both before and after incubation.

It must be concluded that the glucose produced from the excess cellulose is not utilizable by culture 157. No difference has been found between reagent glucose and that produced but not utilized during the breakdown of excess cellulose in old cultures.

Since it has been shown that the reducing sugar found in cultures of 157 was definitely glucose but was not utilizable in the fermentation by this organism, the question of whether it was actually formed by the hydrolytic enzymes of the bac-teria or through nonenzymatic hydrolysis by other factors was investigated. The logical precursor of glucose would be cellobiose because it is a common inter-mediate in cellulose fermentation and is fermentable by culture 157. The con-version of the cellobiose to glucose could conceivably be brought about by the fermentation acids acting at the relatively high incubation temperature to effect a slow nonenzymatic hydrolysis. In order to investigate this possibility an old culture which had fermented 0.1 per cent cellulose was autoclaved without opening. Cellobiose (0.5 per cent) was added, a sample removed, and the flask

again sealed and incubated at 55 C for 26 days. The copper-reducing value initially was 6.4 mg. per ml with a 44 per cent increase on acid hydrolysis, and at 26 days it was 6.1 mg per ml with a 45 per cent increase on acid hydrolysis. These results showed that cellobiose was not converted to glucose by the acids in the culture.

The possibility of glucose arising from cellobiose via the action of cellobiase was next investigated. Medium containing 0.5 per cent cellobiose was inoculated with strain 157 and incubated at 55 C. After 6 days the copper reduction value had decreased from the initial 6.2 mg per ml to 4.0 mg per ml. After 7 more days the value had increased to 5.27 mg per ml. The sugar at the end showed an increase of only 29.8 per cent on acid hydrolysis as compared with the initial 44 per cent, and glucose was demonstrated as the osazone. In view of the previous results in which an autoclaved culture gave no hydrolysis it must be concluded that a cellobiase is present.

Since cellobiose had never been unequivocally demonstrated in a cellulose fermentation, it seemed that the cellobiase shown above might be adaptive and not occur in cultures grown on cellulose. Therefore, the cellobiase activity of a culture grown on cellulose was compared with one grown on cellobiose.

A flask containing 0.15 per cent cellulose and a similar flask with 0.15 per cent cellobiose were inoculated and incubated at 55 C. This concentration of substrate was chosen because it represented the approximate maximum which could be fermented without the accumulation of reducing sugars. Aliquots were removed from both flasks to establish the initial copper reduction value. After 3 days' incubation both fermentations had ceased and the 0.15 per cent cellulose had completely disappeared. At this time the flasks were opened, samples were removed for analysis, and 0.5 per cent sterile cellobiose was added. Samples were again removed for analysis (see below) and the flasks reincubated. A modification of the experiment was also introduced. After the addition of cellobiose to the 0.15 per cent cellulose flask, an aliquot was removed, mixed with toluene, and incubated in parallel with the untreated culture. This was to test for a cellobiase activity in a culture grown solely on cellulose. This seemed necessary because, otherwise, slight growth by the bacteria after cellobiose addition might permit formation of an adaptive cellobiase.

The cultures were terminated after 7 days of reincubation with the cellobiose and analyses performed again. Copper reduction values were determined before and after fermentation with *Escherichia coli*. The difference between these values was used as a rough index of the amount of glucose present. It was demonstrated in separate quantitative experiments that the strain of *E. coli* utilized glucose but not cellobiose. The cultures were autoclaved prior to inoculation with *E. coli* in order to stop any cellobiase activity.

Significant quantities of glucose were formed by cellobiase action whether the cells grew on cellulose or cellobiose (table 5). This shows that cellobiase was present in both the cellulose and cellobiose cultures. The production of glucose in the toluene-treated aliquot was about the same as in the untreated. Therefore the cellobiase must have been formed during the growth on cellulose and was not the result of adaptation after addition of the cellobiose.

This demonstration of a cellobiase in a culture growing on cellulose suggests that cellobiose is a normal intermediate in the breakdown of cellulose and poses the question of why cellobiose had never been found in the quantitative cultures. It seemed possible that enzymatic hydrolysis of the cellobiose might occur during the relatively long period of time which usually elapsed between the complete disappearance of cellulose in the cultures and the initiation of analytical procedures. This might decrease the concentration of cellobiose to the point where it would no longer be easily demonstrable. This was shown to be the case by analyzing a culture with excess cellulose before all the substrate had disappeared. Both glucose and cellobiose were demonstrated by osazone formation and *E. coli* fermentation. But even if the culture was analyzed before all the cellulose had disappeared, the amount of cellobiose was considerably less than the amount of glucose.

TABLE 5

Results of cellobiase experiment

CULTURE	COPPER REDUCTION VALUES					
	Initial	At 3 days		At 10 days		Difference due to activity of cellobiase
		Before cellobiose added	After cellobiose added	Before *E. coli* ferm.	After *E. coli* ferm.	
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
0.15% cellulose..............	0.4*	0.90	7.1	7.7	3.4	4 3
(Toluene added)............				7.2	3.2	4.0
0.15% cellobiose............	1.8	0.82	7.2	7.2	4.3	2.9

* Due to materials not pptd. by $CuSO_4$ but reacting with $Fe_2(SO_4)_3$ or $KMnO_4$.

Pringsheim (1912) found that cellobiase was inactive at temperatures above 67 C. This suggested that more conclusive evidence of cellobiose might be obtained by examining cultures containing an excess of cellulose transferred to this temperature while active fermentation was in progress but before reducing sugars had appeared. Growth soon stopped at 68 C, but cellulose hydrolysis continued. After an incubation period of 7 days osazones were prepared. Only glucosazone was found from the cultures left at 55 C, whereas those transferred to 68 C yielded only cellobiosazone, indicating that cellulase was still active but cellobiase was inactive at this temperature.

DISCUSSION

The experimental data show that under the culture conditions employed the fermentation of small quantities of cellulose (0.15 per cent) occurs without the formation of demonstrable amounts of reducing sugar. If, however, larger amounts of cellulose are present, sugars accumulate during the later stages of the fermentation and after fermentation has ceased.

This is interpreted as indicating that the utilization of cellulose by strain 157 takes place in two steps, hydrolysis and fermentation. In the initial stages of a

culture, fermentation proceeds sufficiently rapidly for all products of hydrolysis to be consumed. This continues until fermentation products have accumulated to the point at which they become inhibitory and fermentation stops. Hydrolysis, however, continues and reducing sugars appear. These sugars have been shown to be cellobiose and glucose, and the evidence indicates that the former is an intermediate stage in the formation of the latter. The breakdown of cellulose to glucose is due to the activity of two different enzymes: (1) a cellulase which hydrolyzes cellulose to cellobiose and is active at 68 C, and (2) a cellobiase which hydrolyzes cellobiose to glucose and is inactive at 68 C.

The most reasonable hypothesis is that the reducing sugars accumulating in the old cultures are intermediate products, not used because of the decreased rate of fermentation induced by toxic end products. This hypothesis is in part invalidated, however, by the inability of culture 157 to use glucose. Not only reagent glucose but also glucose formed by its own hydrolytic enzymes is not attacked. This anomaly necessitates further study before an adequate explanation can be advanced.

SUMMARY

Two pure cultures of thermophilic cellulose-decomposing bacteria have been isolated from natural sources. These constitute the first instances in which cultures of thermophilic cellulose-decomposing anaerobes have been isolated by direct methods which give assurance of culture purity. The method is described in detail.

Quantitative analyses of the fermentation products of the pure cultures acting on cellulose have been made and fermentation balances set up. The products identified are carbon dioxide, hydrogen, ethyl alcohol, formic acid, acetic acid, lactic acid, succinic acid, and glycerol. These account for 70 per cent or less of the cellulose fermented, indicating that unidentified products are also formed.

The hydrolysis of the cellulose continues after fermentation has ceased, with the production of cellobiose and glucose. The cellobiose can be fermented but the glucose cannot. No satisfactory explanation of this anomaly has thus far been found.

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BIOCHEMICAL PROPERTIES OF THE TOXINS OF CLOSTRIDIUM NOVYI AND CLOSTRIDIUM HEMOLYTICUM

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The report of Oakley, Warrack, and Clarke (1947) on the toxins of the *Clostridium novyi* (*oedematiens*) group and of *Clostridium hemolyticum* indicates the complexity of this topic and presents the pertinent historical data. It is known that the toxins *C. novyi* and *C. hemolyticum* involve a lecithinase catalyzing the hydrolysis of lecithin to phosphorylcholine and a diglyceride. In view of the existence of such toxic enzymes as the lecithinases, it was considered possible that the hemotoxins produced by these species might be related to lysolecithin. This phospholipid is a strongly hemolytic substance produced from lecithin by lecithinase A activity and destroyed by lecithinase B hydrolysis to yield nonhemolytic end products. We have demonstrated the ability of lecithinase B to reduce the hemolytic potency of the toxins of *C. novyi*, type B, and *C. hemolyticum*, and this is taken as evidence for the presence of lysolecithin in these toxins.

METHODS

Cultures and media. The strains of organisms used are listed in table 1; their authenticity had been established by one of us previously. They were maintained in the following medium containing desiccated beef heart particles: 10 g tryptone, 2.5 g proteose peptone, 2.5 g peptone, 5 g glucose, 4.2 g Na_2HPO_4 (anhydrous), 0.54 g KH_2PO_4 (anhydrous), 500 ml beef heart infusion, and 500 ml distilled water. Tube cultures in this medium, incubated at 37 C for 24 to 48 hours in Brewer jars, served as inocula for subsequent cultures. The following medium was used for toxin production: 30 g proteose peptone no. 2, 10 g yeast extract, 8.5 g NaCl, 0.1 g $MgSO_4$, 4.2 g Na_2HPO_4 (anhydrous), 0.54 g KH_2PO_4 (anhydrous), 1 g sodium thioglycolate, 100 ml beef heart infusion, and 900 ml distilled water; 0.5 per cent glucose was added after sterilization. Cultures in 3,000-ml Erlenmeyer flasks were incubated at 37 C for 24 to 72 hours in oat jars. Growth was satisfactory with a 1 to 2 per cent inoculum; the final pH was in the range of 6.0 to 6.6. With *C. novyi*, type C, and *C. hemolyticum*, it was necessary to add about 10 g of desiccated heart particles to obtain satisfactory growth in flask cultures. The clear supernatant liquids, obtained by centrifugation after gassing of the cultures had ceased, were saturated with neutral ammonium sulfate. The coagulum which rose was removed, dissolved in a minimum quantity of distilled water, and dialyzed overnight through cellophane against cold running tap water. The nondialyzed material was centrifuged and the supernatant liquid reduced in volume to roughly one-half by vacuum distillation. The concentrated toxic solutions thus obtained were refrigerated and used as needed.

Preparation of lecithinase B. A crude enzyme preparation, obtained by the method of Contardi and Ercoli (1933), was dried from acetone (yield: 8 g per kg of rice bran). Enzyme activity was tested on lysolecithin obtained by the lecithinase A action of snake venom (*Crotalus adamanteus*, the Florida rattlesnake) on soybean lecithin. Commercial lecithin was purified by repeated treatments with ethanol and acetone until a light yellow product was obtained; it was stored under acetone in a refrigerator. About 25 mg of desiccated snake venom were mixed with 500 mg freshly dried lecithin in 50 ml of phosphate buffer, pH 7.0. The mixture was placed in a 45 C water bath for 24 hours and the presence of lysolecithin ascertained by hemolysis tests (positive in dilution of 1:120 with rabbit erythrocytes). In later control experiments for lysolecithin egg yolk suspension also served as a source of lecithin. The yield of lysolecithin

TABLE 1

Strains of Clostridium novyi and Clostridium hemolyticum studied

MC CLUNG COLLECTION NUMBER	NAME OF ORGANISM	TYPE*	PREVIOUS CODE DESIGNATION	SOURCE
842	*C. novyi*	A	N21B	Dr. I. M. Danielson, Lederle Laboratories
41	*C. novyi*	B	B.D. 19	Dr. A. W. Turner, Australia
45	*C. novyi*	B	B.D. Rose	Dr. A. W. Turner, Australia
162	*B. gigas* (*C. novyi*)	(B)	Demnitz 17	Dr. A. Sordelli
50	*C. novyi* (Kraneveld bacillus)	C	Strain I, bacillus osteomylitis; bacillosa bubalorum	Dr. F. C. Kraneveld, Java
808	*C. hemolyticum*		2504	Dr. A. M. Jasmin, Montana State College

* According to Scott, Turner, and Vawter (1934).

was greater under the latter conditions: hemolysis in a dilution of 1:12,800. To test for the activity of the lecithinase B preparation, mixtures of this enzyme and lysolecithin from both sources were made. The lecithinase B preparation reduced the hemolytic potency of the lysolecithin by 32- to 128-fold. It was thus apparent that the rice bran preparation possessed lecithinase B activity.

Indicator techniques for toxin activity. The determination of lecithovitellin (LV) values was performed as follows: Egg saline was prepared by removing aseptically one egg yolk (hen) and mixing with 250 ml of sterile calcium saline, pH 7.6. The latter solution consisted of 0.005 M calcium acetate in 0.85 per cent sodium chloride. To the egg-yolk-saline mixture, 20 g of kaolin or "supercel" were added and the suspension was mixed and centrifuged to clarity. The supernatant was sterilized by passage through a Seitz filter (60-mm pad), stored in a refrigerator, and used within 48 hours. The toxins were diluted in sterile

calcium saline, pH 7.6, all dilutions being made to a volume of 0.5 ml. To each dilution were added 0.5 ml of the egg saline, the tube contents were shaken gently, and the mixtures were placed in a 37 C water bath for 3 hours and then refrigerated overnight. The LV values were graded from negative to four plus; dilutions showing the same appearance as the egg saline similarly diluted were considered negative.

For the determination of hemolytic values, fresh rabbit erythrocytes were washed at least three times with saline, pH 7.0, and used in 3 per cent saline suspensions. The toxins were diluted in sterile calcium saline, pH 6.8, all dilutions being made to a volume of 0.5 ml. To each dilution were added 0.5 ml of the erythrocyte-saline suspension, and the remainder of the procedure was as described for the LV determinations. The hemolytic values were graded from negative to four plus; under no conditions were readings accepted if the control tubes showed signs of hemolysis.

For the determination of lethal values, white mice, weighing about 20 g, were used. The toxins were inoculated intraperitoneally, using 0.5 ml of the diluted material. Three mice per dilution were inoculated and the least amount of toxic preparation causing death of at least two of the three mice within 48 hours was taken as the MLD.

Action of lecithinase B on toxins. To determine the effect of the lecithinase B preparation of the toxins, 100 mg of the enzyme material were suspended in 4 ml of sterile calcium saline, pH 6.8, and 1 ml of toxic solution was added. The mixtures were placed in a 37 C water bath for 24 hours and then tested for LV, hemolytic, and lethality values; suitable controls, without lecithinase B, were always included.

RESULTS

The toxins were tested for LV, hemolytic, and lethality values; the results have been placed in table 2. Also included are the results obtained when 100 mg of lecithinase B were mixed with 5 ml of a 1:5 dilution of the toxins and incubated as indicated above. It can be seen that lecithinase B treatment of the toxins of *C. novyi*, type A, did not affect the LV and hemolytic values. On the other hand, similar treatment of the toxins of *C. novyi*, type B (including *B. gigas*), and of *C. hemolyticum* markedly reduced the hemolytic values of these toxins, without affecting the hemolysis due to the lecithinase (LV-inducing) quota of these toxins. It may be concluded that an additional hemolytic factor is involved in the "hemolysin" of these toxins, and this factor behaves as ly-solecithin. The effect of lecithinase B upon lethality was tested for those toxins shown to contain a lysolecithin hemolysin. Table 2 shows that such treatment caused a decrease in lethal action of the *C. novyi*, type B, and *C. hemolyticum* but not of *B. gigas.* On this point, however, reservation is advocated because the highest lethal dose inoculated was 5 MLD. A lethal dose higher than this value could not be tested because of the problem of dilution. The potency of the lecithinase B preparation was such that it could not be diluted past the levels reported; moreover, the crude enzyme preparation itself is toxic. The

toxin of the Kraneveld bacillus (*C. novyi*, type C) was so weak in its activities that lecithinase B inhibitory action could hardly be evaluated.

A partialy purified preparation of *Clostridium perfringens*, type A, was subjected to the action of lecithinase B. No reduction in hemolytic values was noted with this toxin.

The data for the concentrated toxins presented above were comparative over many trials. Prior to ammonium sulfate precipitation the culture supernatants were all tested similarly, and corresponding, though weaker, reactions were

TABLE 2

*Influence of lecithinase B on the LV, hemolytic, and lethality values of toxins**

TOXIN		WITHOUT LECITHINASE B			WITH LECITHINASE B		
Number	Organism	LV	Hemolysis	Lethality	LV	Hemolysis	Lethality
842	*C. novyi*, type A	1:160	1:320	0.000165	1:160	1:320	Not tested
N241†	*C. novyi*, type A	0.16	0.04	0.015	0.16	0.04	Not tested
41	*C. novyi*, type B	1:160	1:5,120	0.018	1:160	1:320	0.09 (5 MLD) did not kill
45	*C. novyi*, type B	1:640	1:40,960	0.02	1:320	1:640	0.05 (2.5 MLD) did not kill
162	*B. gigas* (*C. novyi*, type B)	1:320	1:2,560	0.20	1:320	1:640	0.4 (2 MLD) killed
50	Kraneveld bacillus (*C. novyi*, type C)	1:8	1:32	Nonlethal	Not tested	Not tested	Not tested
808	Culture supernatant of *C. hemolyticum*	1:1,280	1:81,920	0.0625	1:640	1:1,280	0.25 (4 MLD) did not kill

* Reported as highest dilution giving positive reaction. The lethality values are given as the least amount (ml) causing death.

† Desiccated toxin: values indicate least amount (mg) giving reaction.

generally obtained. Only one exception to this generalization was noted: in the case of *C. hemolyticum* precipitation with ammonium sulfate caused a slight decrease in the hemolytic and lethal values under the prevailing conditions; in this case the data in table 2 were obtained with original culture supernatants.

We wish to indicate here that the terminology used by Macfarlane and Knight (1941) and others with regard to some of the lecithinases is in error. The enzyme responsible for the LV reaction is correctly named "lecithinase D" and *not* "C." This is in accordance with the correct usage by Contardi and Ercoli (1933), the original investigators of this problem. Lecithinase C is a choline phosphatase and catalyzes the hydrolysis of lecithin to yield choline and a phosphodiglyceride.

DISCUSSION

The data presented permit the conclusion that the lecithinase D activities of *C. novyi* and *C. hemolyticum* toxins bear no direct relation to the lethal values. The toxins lacking a lysolecithin hemolysin showed hemolytic values very close to the LV values, indicating the presence of a hemolytic lecithinase. The toxins studied, with hemolytic values in excess of LV values, include those of *C. novyi*, type B, and *C. hemolyticum*. It has been shown that the excess hemolytic values probably are due to lysolecithin, on the basis of lecithinase B inhibition of hemolysis. Moreover, some of the lethal action of the latter two groups of toxins appears attributable to the action of lysolecithin. This is not surprising since Belfanti (1925) showed the marked cytolytic action of lysolecithin in brain, capillary endothelium, and white blood cells in addition to erythrocytes.

Oakley, Warrack, and Clarke (1947) demonstrated the existence of a toxin common to *C. novyi*, type B, and *C. hemolyticum* toxins; this was called beta toxin and acted as a hemolytic, necrotizing lecithinase. In our study the occurrence of the lysolecithin hemolysin as a common toxic component is noted. The function of lysolecithin as an antigenic component can be explained on the basis of the assumption that this phospholipid occurs in nature as a lysolecithoprotein, in the same fashion as its parent substance, lecithoprotein. This assumption satisfies the serological data as well as the knowledge that the toxic component was precipitated by ammonium sulfate saturation and is not dialyzable through cellophane. Demnitz (1934), Sordelli and Ferrari (1937), Keppie (1944), Hayward and Gray (1946), as well as Oakley, Warrack, and Clarke (1947), supplied evidence to show that not all the hemolysins of *C. novyi*, type A, and *C. novyi*, type B, were identical. Moreover, there were interrelationships between the hemolysins of *B. gigas* (*C. novyi*, type B) and *C. hemolyticum*. We suggest that a common antigen of these toxins is the lysolecithin hemolysin described above.

The possibility that the rice bran preparation contained substances other than lecithinase B is appreciated. It may be admitted, however, that this enzyme was present and that certain differences were noted with some of the toxins. The differences reported appear to be significant and are not contradicted by existing knowledge. As for the hypothetical substances involved, they must be viewed as constituting such experimental risks as are common in enzyme chemistry.

SUMMARY

With lecithinase B, the specific hydrolytic enzyme acting on lysolecithin, as an indicator system, it was found that the toxins of *Clostridium novyi*, type B, and *Clostridium hemolyticum* contained a hemolysin showing the properties of lysolecithin. Treatment of these toxins with lecithinase B reduced lethality. The toxins of *C. novyi*, type A, and *Clostridium perfringens*, type A, do not contain such a hemolysin. The lecithinase D activities of *C. novyi* and *C. hemolyticum* toxins bear no relation to the lethal values.

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INDUCED BIOCHEMICAL MUTANTS OF AZOTOBACTER AGILIS

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Through the intense study of biochemical genetics in recent years an insight has been gained into the mechanism of gene action, which in conjunction with previously known facts has made it possible to investigate the existence of gene-like determinants in organisms that do not lend themselves to ordinary genetical analysis. The application of these developments has opened up the previously almost nonexistent field of bacterial genetics, and on the basis of recent evidence (Gray and Tatum, 1944; Lederberg, 1947) it is already fairly well established that at least some bacteria possess organizers that correspond to the genes in higher forms.

Bacteria, like higher organisms, are characterized by a great hereditary stability, even with regard to characteristics that are only potentially existing, such as adaptive enzymes. This makes it necessary to assume the existence of a precise mechanism whereby the parental characteristics are exactly duplicated in the offspring. A certain degree of mutability is still exhibited, the direction of which is as a rule independent of external factors, and new characters thus established are passed on with the same precision as the original ones. The frequency of appearance of new forms is increased by destructive agents that increase the mutation rate in higher organisms. And, as far as has been established, the function of the bacterial hereditary determinants seems to be the same as that of the genes in higher forms. This is indicated by the fact that artificially induced variants with specific growth factor requirements are obtainable by the same methods as in sexually reproducing organisms (Gray and Tatum, 1944). Presumably these variants are unable to carry out a particular chemical process in a synthetic reaction chain like similar mutants of *Neurospora*, although this has not been specifically demonstrated.

The work presented in this paper was started early in 1944, when the possibilities of new developments in the field of bacterial genetics were just beginning to be realized. But even at that time a close resemblance between the basic hereditary mechanisms of bacteria and higher forms seemed so probable that it was decided to make an attempt to isolate not only the already known types of biochemical mutants, but also variants with specific genetic blocks in some major metabolic pathways, which then could be studied with the aid of such mutants. The main interest was in the oxidative dissimilation of organic compounds. Although variants of this type had not been obtained artificially, they were known to occur in nature, so that no good reason was evident why they could not be produced. But since it was not certain that such mutations could be isolated from irradiated material, the process of nitrogen fixation was selected as a second choice, as it is known to be dispensable in the presence of nitrogenous compounds.

Azotobacter offers suitable material for the study of the mechanisms of both oxidative dissimilation and nitrogen fixation and was therefore chosen for this investigation.

<div align="center">MATERIALS AND METHODS</div>

The organism employed in this study was *Azotobacter agilis*, strains 4.4 and 4.5, obtained from Professor C. B. van Niel. After several single colony isolations uniformity in colony size was attained, and subsequently all experiments were started from single colonies. *A. agilis* is more satisfactory than other *Azotobacter* species for these experiments because the cells are more easily separated and more easily identified by microscopic examination.

The following basal medium (medium 1), made up in distilled water, was used when nothing else is specified: 1 per cent (vol.) ethyl alcohol, 0.1 per cent K_2HPO_4, 0.02 per cent $MgSO_4$, 0.01 per cent $CaSO_4$, 0.003 per cent $FeSO_4$, and 0.0002 per cent $NaMoO_4$. The pH was adjusted to 7.2 with hydrochloric acid. In solid media 1.5 per cent agar was included. The incubation temperature was 28 to 30 C. Liquid media were usually agitated on a horizontal shaker to ensure uniform and rapid growth.

For the isolation of irradiated bacteria a partially selective medium of the following composition was employed (medium 2): 1.0 per cent (vol.) ethyl alcohol, 0.01 per cent glucose, 0.02 per cent yeast extract (Difco), 0.04 per cent K_2HPO_4, 0.02 per cent $MgSO_4$, 0.01 per cent $CaSO_4$, 0.003 per cent $FeSO_4$, 0.0002 per cent $NaMoO_4$, 1.0 per cent (vol.) of a 0.04 per cent aqueous bromthymol blue indicator solution; the pH was adjusted to 7.2. The important features of this medium are: (a) alcohol is the only abundant energy source, so that colonies unable to utilize it for growth will appear small; (b) the low buffering capacity and the indicator permit the formation of a small amount of acid or alkali to be detected; (c) the total amount of nitrogen, added in the form of yeast extract, is so standardized as to permit only limited growth in the absence of nitrogen fixation; (d) the presence of the yeast extract should permit development of mutants requiring growth factors. The use of this selective medium made the detection of variants with disturbances in the nitrogen fixation or alcohol oxidation mechanisms more probable, but did not discriminate against any types of mutants. Thus the probability of finding growth factor variants or mutants unable to oxidize glucose was the same as if colonies were tested at random.

Mutations were induced by X-ray treatment of 1 ml of a 40-hr culture in the basal medium. The irradiation was carried out with an oil-cooled X-ray tube operated at 200 kilovolts and 15 milliamperes. The distance from the target was 47 cm when the dosage was 1,250 roentgens per minute. The total exposure was 150,000 roentgens, which killed approximately 99.99 per cent of the cells. The remaining viable cells were plated out on the surface of medium 2 and incubated for 3 to 4 days. Small and otherwise aberrant colonies from these plates were tested for growth on medium 1 with alcohol alone or with glucose alone. As a rule these tests were carried out on solid media to avoid loss of unstable variants.

If abnormal behavior was again observed, further tests were carried out in accordance with the suspected characteristics.

Growth was measured with the Evelyn photoelectric colorimeter and expressed as $2 - \log G$.

RESULTS

Several physiological mutants of *A. agilis* were obtained by isolation from X-ray-treated cultures. The data are not extensive enough to warrant a statement as to the mutation frequency, particularly since the methods do not ensure detection of all mutants, but the indications are that the frequency is in the same order of magnitude as in other bacteria. The data from the irradiation experiments are summarized in table 1.

As shown in the table almost 8,000 single cell isolations were made from irradiated material, but only 383 colonies were specifically tested for altered

TABLE 1

Results of X-ray treatment of A. agilis

EXPERIMENT	X-RAY DOSAGE	NO. AT START	PERCENTAGE KILLING	COLONIES ISOLATED	COLONIES SPECIFICALLY TESTED	PHYSIOLOGICAL STRAINS
1*	1.1×10^5	2×10^6	99.92	1,500	93	1
2	—	1.5×10^6	99.40	4,950	27	0
3	1.5×10^5	1.5×10^6	99.997	26	1	1
4	—	5×10^6	99.95	1,338	192	0
5	1.5×10^5	5×10^6	99.996	70	70	1

* A Coolidge type X-ray tube, operated at 100 kv and 5 ma, was used for this experiment.

physiological characteristics. The majority of these were small or otherwise aberrant colonies.

Of great interest is the high degree of mutability with regard to colony form and size and possibly other characteristics that is exhibited on subsequent transfer of colonies from X-ray-treated material. A further study of this latent variation would certainly be pertinent, but little attention was given to it in this work, since its significance was not realized at the outset. In all probability this phenomenon is a manifestation of the same mechanism as the latent variation in *Escherichia coli*, which has been reported by Demerec (1946). The possibility seems very attractive that the same factors might also be responsible for the instability of some of the biochemical strains.

Morphological variants, involving colony form and color, are quite frequent. The parent strain forms a greenish-yellow water-soluble pigment, but approximately 20 per cent of the viable irradiated population in each experiment were no longer able to form the pigment. Similarly, a high proportion of rough colony variants, up to 40 per cent, was found in the treated material, whereas the original strain consisted entirely of smooth colony variants.

Physiological variants other than colorless ones are less frequently encountered, but they are of the same kind as those already described in other organisms in the sense that they lack the ability to form certain compounds required in the normal metabolism.

A discussion of the biochemical characteristics of the mutants is present below.

Pigment variants. As already mentioned, among the most common variants are those lacking the ability to form the diffusible greenish pigment that is characteristic of *A. agilis*. These mutants appear like the normal strain when grown in shaken liquid cultures without combined nitrogen, but growth is very slight on solid media of the same composition and is slow in stationary test tubes. Solid media supplemented with yeast extract support good growth, and at least some of these variants give a response to added ammonia, which inhibits the normal strain
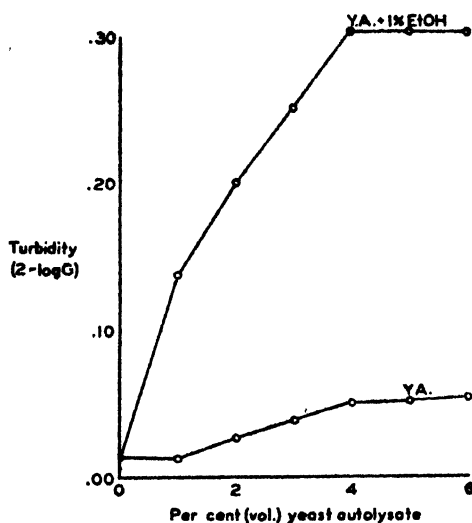
Figure 1. Growth of strain A3 in medium 1 in the presence and in the absence of ethyl alcohol with different amounts of yeast autolysate (3 days).

to some extent. The slight growth obtained on the solid basal medium appears glossy, and microscopic examination reveals large, refractile globules that fill the cells. Motility is absent. These mutants are rather stable and have never been observed to revert. The fact that they differ from the other kinds of physiological mutants, occurring more frequently and being more stable, suggests that they may be formed by a different mutation path.

Variants requiring growth factors. The first clear-cut nutritional variant isolated, strain A3, was so unstable as to make further characterization difficult. For this reason the particular growth factor required was never identified, but it was definitely established that something present in yeast extract was needed, growth on alcohol being proportional to the amount of yeast extract supplied, as is shown graphically in figure 1.

The second physiological mutant, strain A5, was also unstable, possibly no less so than A3; but since more experience had been gained at that stage, this variant was characterized as quickly as possible without any transfers except to the first plate, where growth was very uniform. The colonies from this plate were then
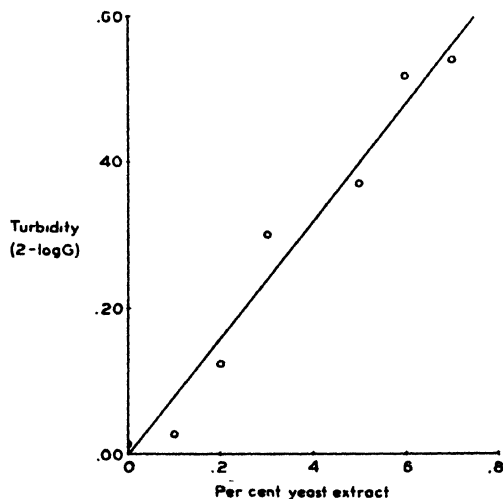
Figure 2. Growth of strain A5 on medium 1 containing 1 per cent ethyl alcohol and different amounts of yeast extract (Difco) Incubation time, 3 days.
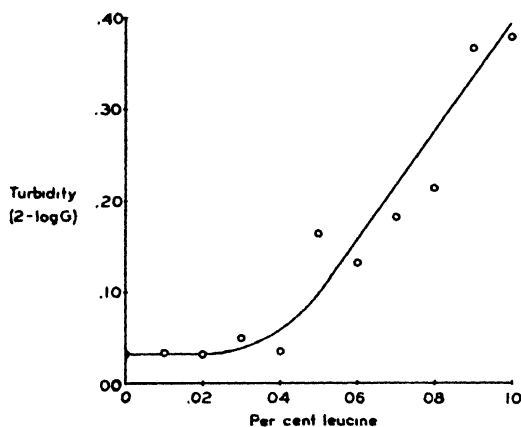
Figure 3. Growth of strain A5 in medium 1 supplemented with L-leucine. Incubation time, 4 days.

used for all the subsequent tests. Growth was proportional to the amount of yeast extract added as shown by figure 2, and casein hydrolyzate seemed to contain roughly three times as much of the required substance as the yeast extract. Finally the amino acid leucine was identified as a growth factor for this strain, as is indicated in figure 3.

When the first transfer of strain A5 to 0.5 per cent yeast extract was made, growth was very homogeneous, so that all the colonies appeared alike, slightly rough, and average in size. From then on difficulties were encountered because of instability, and a uniform culture with the original characteristics could not be re-established. Growth occurred even on the basal medium after a long initial lag period, during which the cells were filamentous, similar to those shown by Den Dooren de Jong (1938). When adapted, these elongated forms transformed into the ordinary spherical cell type.

Variants with disturbed energy metabolism. The last physiological variant isolated, strain A13, was detected as a colony unable to utilize glucose for growth. When grown on alcohol, it was indistinguishable from the parent strain. A high degree of stability was indicated by the finding that in a liquid medium containing both alcohol and glucose the total growth corresponded only to the alcohol,

A4.4   A13   A4 4   A13
ETHYL ALCOHOL   GLUCOSE
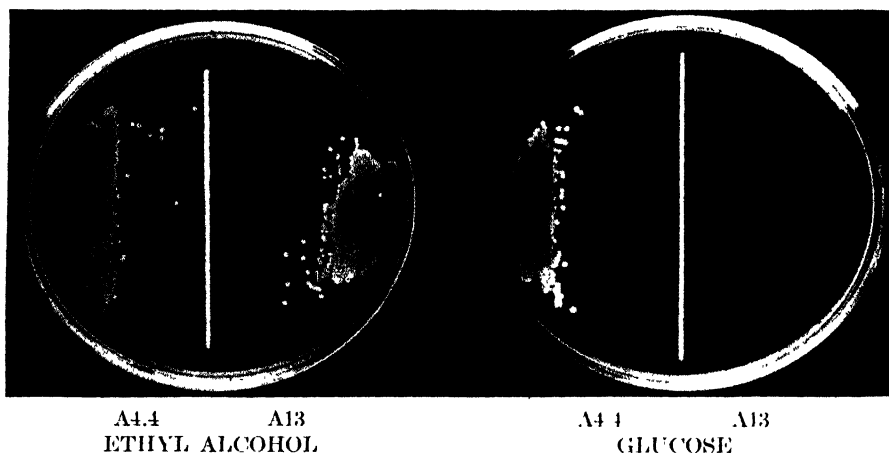
Figure 4. Growth of strains A4.4 and A13 on medium 1 containing alcohol or glucose.

showing that not a single reversal occurred during the growth of millions of cells. However, reversion was effected several times by long-continued shaking in liquid media, and the resulting organism was identical with the original strain, as far as substrate utilization and morphological characters were concerned. These facts, namely, that reversal can occur and that all the characteristics of the mutant revert at the same time, indicate that only a single alteration is responsible for the disturbances in the energy metabolism of this strain.

The biochemical characteristics of strain A13 will be described elsewhere. It has lost the ability to oxidize pyruvate to acetate, which seems to be the main channel of pyruvate breakdown in the parent strain. A13 is unable to grow on any substrate that is arrested at the pyruvate stage, since acetate or its derivatives are essential for development. Growth is supported only by alcohol, acetate, and malonate. The following compounds used by the parent strain cannot serve as growth substrates for the mutant: glucose, fructose, gluconate,

tartarate, *trans*-aconitate, *cis*-aconitate, α-ketoglutarate, succinate, fumarate, malate, lactate, and pyruvate.

Figure 4 is a photograph of the growth of the original and mutant strains with alcohol or glucose as an energy source.

ACKNOWLEDGMENT

SUMMARY

It has been demonstrated that X-ray-induced mutants are obtainable in *Azotobacter agilis*. Morphological variants, involving colony form and color, occur frequently, and physiological mutants have also been produced.

A special search was made for variants with disturbed energy metabolism, and a rather stable mutant was discovered which, because of a complete genetic block, is unable to convert pyruvate to acetate. Since acetate or its derivatives are essential for growth, this mutant is not able to develop on substrates such as glucose and succinate, which are arrested at the pyruvate stage.

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FUNGI CAPABLE OF GROWING IN STRONGLY ACID MEDIA AND IN CONCENTRATED COPPER SULFATE SOLUTIONS

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It is well established that the bacterium *Thiobacillus thiooxidans* is capable of growing in very strong mineral acid solutions and of producing, from elementary sulfur, sulfuric acid solutions more concentrated than 1 N and a reaction considerably below pH 1. This organism had been considered unique in its tolerance to acid until Starkey and Waksman (1943) demonstrated that certain fungi, *Acontium velatum* Moore, and an unidentified dematiaceous mold were able to develop in a medium initially as acid as 2.5 N sulfuric acid and in a normal sulfuric acid solution saturated with copper sulfate.

We were able to confirm the tolerance to extreme acidities of some fungi with three isolates and tolerance to copper sulfate with two of them. The first isolate, no. 7752, was obtained from a bottle of N/5 sulfuric acid reagent, the second, no. 9010, from soil rubbish picked up from an athletic running track, and the third, no. 9024, from another sample of standard sulfuric acid reagent.

Numbers 7752 and 9024 are possibly the same as Starkey and Waksman's unidentified dematiaceous mold. It has been tentatively identified as *Trichosporon cerebriforme* (Kambayashi) Ota by Dr. R. L. Bouthilet. Difficulties in getting authentic cultures for comparison or original descriptions published in Japan have made it impossible for him to make an absolute identification as yet. The third isolate died out before it was identified.

The first experiment demonstrates that when *T. cerebriforme* (which breaks up readily into what we interpreted as arthrospores, but Starkey and Waksman as chlamydospores) is inoculated, in amounts so small as to be invisible in the culture, into a medium consisting of 1, 1.5, or 2 N solutions of sulfuric acid plus 0.1 per cent glucose and 0.1 per cent peptone, a growth results easily visible as dark, almost black, globose masses of hyphae. Growth was evident in about 10 days with the 1 N acid solution, but prolonged incubation was necessary to obtain the maximum yields. The incubation was at room temperature.

The second experiment was in part a confirmation of the first. Narrow-mouthed bottles of about 400-ml capacity, half filled with media, were used. Some were sealed with glass stoppers; others were plugged with cotton wool. The media in the second experiment were essentially the same as in the first, except that some were more strongly acid, and some were not autoclaved. The normalities of the media were determined before and after incubation. Following

the prolonged incubation the viability of the cultures was tested by plating on 4 petri plates, using potato glucose agar, 1-ml portions of water blanks, each blank containing 4 loopfuls of the thoroughly shaken culture. Such small inocula do not prove that the mold was no longer alive in those cultures in which it was

TABLE 1

Growth of two isolates of molds in acid media

NUMBER OF ISOLATE	NORMALITY OF SULFURIC ACID	AGE OF GROWTH	AMOUNT OF GROWTH
		days	
7752	1.0	67	Fair
7752	1.47	67	Fair
7752	2.04	103*	Light
7752	2.04	103*	Fair
9024	1.0	58	Heavy
9024	1.47	58	Heavy
9024	2.04	58	Light

* No visible growth in 67 days.

TABLE 2

Growth and viability of molds on prolonged incubation in acids

CULTURE NUMBER	STOPPER	AUTOCLAVED	NORMALITY OF ACID		INCUBATION	GROWTH	PLATES POSITIVE
			Initial	Final			
					days		
7752	Cotton	+	1.00	1.46	678	+	4
7752	Cotton	+	1.47	2.32	678	+	4
7752	Cotton	+	2.0	2.92	678	+	0
7752	Glass	−	2.04	2.20	713	+	0
7752	Cotton	+	2.49	3.52	678	−	0
7752	Glass	−	2.56	2.76	724	+	0
9010	Cotton	+	1.0	1.94	522	+	4
9010	Cotton	+	1.47	4.23	522	+	1
9010	Glass	−	2.0	2.05	569	+	3
9024	Cotton	+	1.0	2.25	669	+	0
9024	Cotton	+	1.47	—	669	+	0
9024	Cotton	−	2.04	4.93	669	+	0
9024	Cotton	+	2.04	3.98	669	+	0
9024	Cotton	−	2.49	4.58	669	−	0
Control	Cotton	+	2.0	2.84	678	−	0
Control	Cotton	+	2.49	3.51	678	−	0
Control	Glass	−	1.0	1.01	679	−	0
Control	Glass	−	1.47	1.52	679	−	0
Control	Glass	−	2.0	2.07	679	−	0

not found, but do demonstrate that it was still viable in those which yielded positive results.

Table 2 shows that one of the three strains produced a visible growth in 2.5 N sulfuric acid, and all three did so in 2.0 N sulfuric acid. It was demonstrated that

there was a tendency for the isolates to die out in prolonged incubation, especially where the acidity was allowed to increase by evaporation.

A single experiment with 1 N hydrochloric acid with the addition of 0.1 per cent each of glucose and peptone indicated that cultures of *T. cerebriforme* grew in other mineral acids of high titer.

It was further found that a solution of 280 grams of $CuSO_4 \cdot 5H_2O$, 1 gram of glucose, and 1 gram of peptone per liter of 1 N sulfuric acid allowed good development of both isolates of *T. cerebriforme*. This also confirms the amazing findings of Starkey and Waksman, findings which, incidentally, have been doubted by certain individuals.

Although pH determinations were made, they are not reported. The great inaccuracies of measurements with the glass electrode of hydrogen ion activities at the pH values which were less than 1, and indeed in many cases less than 0
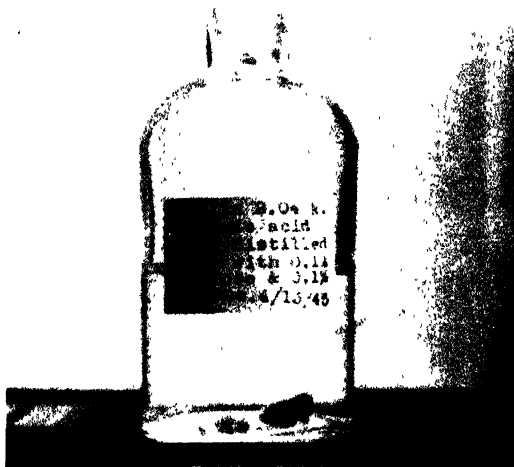
Figure 1 Growth of mold in 2 N sulfuric acid solution.

are such that determinations of normalities in these poorly buffered solutions are more meaningful.

SUMMARY

A fungus provisionally identified as *Trichosporon cerebriforme* was found to grow in 1, 1.5, 2, and 2.5 normal sulfuric acid fortified with the addition of 0.1 per cent glucose and 0.1 per cent peptone. Another isolate of the same species and an unidentified mold grew at normalities of 1, 1.5, and 2. Both isolates of *T. cerebriforme* grew in a solution of 280 g $CuSO_4 \cdot 5H_2O$, 1 g glucose, and 1 g peptone per liter of 1 N sulfuric acid.

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ENZYMIC ACTIVITY OF BACTERIOPHAGE-CULTURE LYSATES

I. A Capsule Lysin Active against Klebsiella pneumoniae Type A[1]

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Certain strains of bacteriophage produce plaques surrounded by zones in which the bacteria are not dissolved but in which the bacterial film has become more translucent and thinner than the surrounding normal bacterial growth. In contrast to the plaques proper, which do not noticeably increase in diameter after the first few hours of bacterial growth, these zones gradually increase in width as incubation is prolonged (Asheshov, 1924; Sertic, 1929a,b; Boulgakow, 1929; Gratia, 1937; Rakieten et al., 1940). The bacteria within the zones are viable and may, upon transfer, give rise either to normal growth when not contaminated by phage (Sertic, 1929a) or, usually, to secondary, resistant cultures if the phage has diffused extensively from the plaques (Rakieten et al., 1940).

The mechanism of the zone production seems first to have been studied by Sertic (1929a,b). Working with a zone-producing *Escherichia coli*, bacteriophage system, this author obtained, free from bacteria and bacteriophage, a "lysin" that reproduced the zone in either living or chloroform-killed films of the *E. coli* culture. The activity of this lysin was proportional to its concentration, and it was not reproduced in the absence of the phage. In later publications (Sertic and Boulgakow, 1937; Sertic, 1937), it was demonstrated serologically and by phage and lysin sensitivity tests that the lysin specifically removed an antigenic membrane from the surface of the *E. coli* cell. The lysin caused no apparent harm to cell vitality, and progeny of treated cells again produced the antigenic coating in the absence of the lysin.

EXPERIMENTAL DATA

In the present paper studies on the demonstration, on the separation, and on some of the characters of a lysin (to be referred to hereafter as C lysin) produced by a *Klebsiella pneumoniae*, bacteriophage system are reported. One strain of *Klebsiella pneumoniae*, type A (108M), has been used in most of the experiments. This strain produces a well-defined capsule and is virulent for mice.

The bacteriophage used was isolated in 1943 from sewage plant effluent and was obtained in pure line by successive single plaque isolations. It has been maintained in the form of filter-sterilized lysates at refrigerator temperature. This phage strain, designated as "phage MA8," appears to be type-specific in its host selectivity: of the encapsulated strains thus far tested, the 5 type A Friedländer cultures are all susceptible to the lytic action of this phage, whereas the 1 type

B *K. pneumoniae* culture, the 1 type C *K. pneumoniae* culture, and the 2 encapsulated *Aerobacter aerogenes* strains are resistant. The phage also fails to lyse Friedländer strains in the smooth culture phase even though these cultures have been obtained from type A strains. The plaques produced by this phage in culture films on 1.5 per cent agar media average less than 1 mm in diameter and are surrounded by marked zones that increase in diameter on continued incubation. Both in its type specificity and in zone productivity this phage is like the *Klebsiella* phages described by Rakieten *et al.* (1940).

Production and Titration of C Lysin

Broth composed of tryptose 2 per cent, glucose 0.25 per cent, NaCl 0.5 per cent, Na_2HPO_4 0.25 per cent, and bromthymol blue (1.6 per cent alc.) 0.1 per cent and adjusted to pH 7.5 was used in the routine production of phage lysates. This broth was inoculated heavily with a young broth culture of strain 108M, infected with phage MA8, and incubated until mass lysis had occurred. Within the limits concomitant with mass lysis, the greater the ratio of bacterium to phage inocula the greater the yield of lysin. The glucose in the medium hastened bacterial growth and mass lysis and resulted in increased yields of C lysin. The development of excess acidity during incubation was prevented by neutralization from time to time with N NaOH solution. Lysates were sterilized by filtration through porcelain (Selas, 02 porosity) filter candles and stored at refrigerator temperature.

The presence of C lysin in these phage filtrates can be tested by the spot inoculation of loopfuls of filtrates onto the surface of adult growth films of the susceptible organism, followed by incubation. Under these conditions no plaques are formed, but zone-type clearings appear in the bacterial growth at the sites of inoculations.

Separation of lysin and phage. Phage inactivation without lysin destruction has been achieved by heating filtrates to 70 C for 30 minutes or by adding sufficient formalin to filtrates to give a final formalin concentration of 0.5 per cent. By these procedures the lytic activity of the phage is destroyed. However, phage particles presumably are still present, and the possibility remained that zone production might be another more stable manifestation of direct phage action on the cell. In order to prove lysin activity to be independent of the phage, it seemed necessary to obtain a phage-free zone producing filtrate.

Since ultrafiltration had been used successfully by Sertic (1929a,b) for the separation of phage and lysin, it was decided to use this method in the present work. Graded porosity ultrafilter membranes were prepared from alcohol-ether solutions of collodion (Mallinckrodt, U.S.P. XI) using essentially the technique of Asheshov (1933a,b). However, the batch of ethyl acetate at hand failed to induce sufficient spread in membrane porosity, and it was necessary to find a substitute. Elford had reported (1931) that distilled water in gradually increasing concentrations up to 5 per cent produces uniform increase in membrane permeability. This reagent was found to produce satisfactory porosity modifications in the membranes.

For use, the membranes are backed by filter paper, supported on a perforated aluminum disk, and inserted in a Seitz filter apparatus in the position of the usual filter pad. Leakage is prevented by two rubber washers, one placed beneath the aluminum disk and the other on top of the collodion membrane. The top of the filter apparatus is then clamped securely in place and the filter is ready for use.

The membranes were graded on the basis of the time required for 1 ml of distilled water to pass through 1 cm² of membrane area at 50-cm negative pressure (Asheshov, 1933b).

Filter-sterilized phage lysate was passed through each membrane of a series, and the ultrafiltrates were tested for phage activity and for ability to produce zone-type clearings in surface culture growth of *Klebsiella pneumoniae* 108M. An ultrafiltrate was considered to be phage-free when it was not possible to demonstrate the presence of phage by the plaque method following the plating of 0.1-ml volumes of the filtrate of a young broth culture inoculated with 1.0 ml of ultrafiltrate and incubated overnight before filtering. Membranes with a rate of flow of 180 seconds or less usually allowed passage of both the phage and the lysin, whereas membranes with a rate of flow of 180 seconds or slower held back the phage and allowed passage of the C lysin. Phage-free, C-lysin-active ultra-filtrates will subsequently be referred to as "C lysin UF."

C lysin is apparently nontoxic to the bacterial cells. Strain 108M grows equally well in broth, in broth containing C lysin UF (20 per cent), and in broth containing heat-inactivated C lysin UF (20 per cent).

Titration of C lysin. The zone type of clearing produced by the lysin furnishes the basis for a simple yet fairly accurate method of titrating the C potency of ultrafiltrates. Twofold or fourfold serial dilutions of such preparations are tested for their ability to produce clearing in a film of bacterial growth under the following standardized conditions:

A sterile petri plate is placed on a plane surface and a 25-ml portion of proteose peptone no. 3 agar[*] is poured into it and allowed to harden. The entire surface of this plate is then inoculated with strain 108M by covering the agar with a turbid broth culture, the excess fluid being removed by means of a capillary pipette. The glass petri plate top is then replaced by a top of porous clay, and the plate is incubated (on the plane surface) until the surface of the agar appears dry. The glass plate top is resubstituted, and the plate is inverted and incubated at 37 C overnight. This technique results in an even film of growth on the agar and is reproducible.

In the titrations, one 3-mm loopful of each ultrafiltrate dilution is placed on the agar surface growth and the plate is incubated at 37 C for 18 hours. The greatest dilution causing maximum clearing is read as the end point. C strength can also be estimated, somewhat less accurately but more rapidly, by noting the rate of zone-clearing production of undiluted ultrafiltrates in these standard agar plate cultures. The C lysin UF used in the following experiments had a titer of 1 to 32.

[*] Proteose peptone no. 3 (Difco) 2.0 per cent, NaCl 0.5 per cent, Na₂HPO₄ 0.25 per cent, agar 1.5 per cent, and H₂O, distilled, 1,000 ml.

The Capsule-dissolving Action of C Lysin

The thinness and translucence of the bacterial film composing the zone and the fact that smooth (nonencapsulated) culture variants of *Klebsiella pneumoniae* produce thinner and less opaque growths than their mucoid precursors suggested that the lysin caused decapsulation of the cells. This hypothesis was demonstrated to be correct by several complementary techniques using actively growing cultures, killed cell suspensions, or both.

Antigenic analysis: in vitro experiments. The 108M cells are agglutinated by type A antiserum but fail to be agglutinated by an antiserum against its S culture phase variant. Since type specificity is associated with encapsulation and since the only known structural difference between the M and S culture phase cells is the presence of the capsule around the M cells, it seemed reasonable to expect that dissolution of the capsule would result in a loss of agglutinability in pure M antiserum and a gain of agglutinability in S antiserum. This hypothesis was tested as follows:

TABLE 1

Effect of C lysin on the antigenic structure of strain 108M

CULTURE GROWN IN PRESENCE OF	ANTI-SERUM	SERUM DILUTIONS											CON-TROL
		20	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	
C lysin	M	—	—	—	—	—	—	O	O	O	O	O	—
	S	F	F	G	G	G	G	G	G^f	G^f	G^f	—	—
Heated C lysin	M	F	F	F^f	G	—	—	O	O	O	O	O	—
	S	—	—	—	—	—	—	—	—	—	—	—	—
No C lysin	M	F	F	F^f	—	—	—	O	O	O	O	O	—
	S	—	—	—	—	—	—	—	—	—	—	—	—

M = monospecific anti-108M-serum.   — = no agglutination.
S = anti-108S-serum; titer 1:10,240.   O = not done.
F = floccular agglutination.   f = finely.
G = granular agglutination.

An anti-S rabbit serum was prepared against an S sector variant culture of 108M. It agglutinated the homologous culture to a titer of 1:10,240 but failed to agglutinate strain 108M. A monospecific antiserum was prepared by the adsorption of the noncapsular agglutinins from an anti-108M rabbit serum by means of S sector variant cells.

In the first test of the effect of C lysin on antigenic structure, the following experiment was performed: Strain 108M was grown in tryptose glucose broth and in tryptose glucose broth to which had been added either C lysin UF or heat-inactivated C lysin UF (25 per cent by volume). After 6½ hours' incubation at 37 C, formalin was added to each culture to give a 0.5 per cent final concentration. The cells were then washed by centrifugation, suspended in 0.5 per cent formalinized physiological saline, and set up against varying dilutions of monospecific anti-M-serum and of anti-S-serum in tube agglutination tests.

Incubation was carried out in a 37 C water bath for 18 hours. Results were read macroscopically. The data are summarized in table 1. These data clearly

show that the growth of 108M in the presence of C lysin results in a loss of type-specific agglutinability of the cells and that such cells have become agglutinable by S phase antiserum.   These results have been confirmed by exposing formalinized 108M cell suspensions to C lysin UF and to heat-inactivated C lysin UF followed by agglutinability tests.   Here, also, the C-lysin-treated cells had become inagglutinable by monospecific M antiserum but were agglutinated in high titer by the S antiserum, whereas the heat-inactivated C control suspension had retained its type specificity and its inagglutinability in S antiserum.

The gain of agglutinability of the C-treated 108M cells in S antiserum was again demonstrated in the following experiment: Strain 108M was grown in tryptose glucose broth to which had been added S antiserum (1 per cent by volume) and either C lysin UF or heat-inactivated C lysin UF (20 per cent by volume).   In the cultures containing active C lysin, growth occurred in the form of granular clumps (typical "O" type agglutination), whereas in the cultures in which inactivated C lysin was present there developed an even turbidity.   The possibility that M to S culture phase dissociation might have occurred was ruled out by transferring a loopful of the granular growth from the C lysin tube to a tube containing inactivated C lysin and S antiserum.   The resulting growth occurred evenly dispersed throughout the medium.

In vivo experiments.   In order to determine whether C-treated cells had also lost the ability to stimulate the production of type specific antibodies, the following experiment was performed: Culture 108M was grown in 20 per cent C lysin UF tryptose glucose broth until the culture was quite turbid.   The culture was then formalinized.   The cells were washed twice by centrifugation and resuspended in 0.5 per cent formalinized physiological saline.   By the tube technique this suspension failed to be agglutinated by the monospecific M antiserum in a dilution of 1:20 but was agglutinated to a titer of 1:5,120 in the S antiserum. Two rabbits were then each given a series of 6 intravenous injections with increasing doses of the suspension.   Nine days after receiving the last injection trial bleedings were made and the sera were tested for agglutinating activity against the formalinized C-lysin-treated suspension and against a formalinized untreated suspension of culture 108M.   Although agglutinins against the C-lysin-treated cells were present to a high titer (1:8,192 and 1:16,384, respectively), these antisera failed to agglutinate strain 108M even in a dilution of 1:2.   Judged by the agglutination test, C-lysin-treated cells are no longer able to stimulate type-specific antibodies in the rabbit.

Cell volume determinations.   Dissolution of the capsule should result in a marked reduction in cell volume.   The following experiment was performed in order to determine whether C lysin treatment results in a reduction in cell volume. Aliquots of a formalinized (0.5 per cent) young, turbid, tryptose glucose broth culture of 108M were subjected to C lysin UF and to boiled C lysin UF (25 per cent by volume) for 4 hours at 37 C.   Microscopic cell counts were made on suitable dilutions of each suspension utilizing a Zeiss bacterial counting chamber, and mass cell volumes were measured by the Hopkins technique (Hopkins, 1913). The data thus obtained are summarized in table 2.

As the result of C lysin treatment the total cell volume of a 3-ml aliquot was

reduced from 0.060 ml to 0.015 ml (a 75 per cent reduction), and this reduction was due, as the cell counts indicate, to a decrease in average cell size rather than to a reduction in cell numbers. Similar experiments have confirmed that, under these conditions of culture, a 75 per cent decrease in cell volume is about average for strain 108M following exposure to C lysin.

Phage susceptibility test. Sertic (1937) found that the lysin studied by him rendered the *E. coli* cells subject to the lytic action of certain other phages against which this culture was normally resistant. He attributed this developed susceptibility to the removal by the lysin of a surface coating from these cells, rendering the bodies of the microbes available to other bacteriophagic lysins. We have in our collection a phage which is lytic for the S sector variant culture of strain 108M but which fails to lyse the parent culture. It seemed reasonable to expect that if C lysin decapsulated these type A cells, they might be subject to lysis by the S phage. This was found to be the case. No lysis of strain 108M occurred in tryptose glucose broth containing heat-inactivated C lysin UF (20 per cent by volume) and phage S8. However, when this medium containing like concentrations of C lysin UF and phage S8 was inoculated with an amount of

TABLE 2

Effect of C lysin on the cell volume of strain 108M

CELLS EXPOSED TO	COUNT NO.	DIRECT CELL COUNTS 1/25 SQ MM AREAS	AVERAGE COUNT PER 1/25 SQ MM		APPROX. NO. CELLS PER ML	MASS CELL VOLUME PER 3 ML
						ml
C lysin	1	72, 83, 91, 88, 90, 96,	87	80	2,000,000,000	0.015
	2	65,64, 76, 65, 101, 66	73			
Heated C lysin	1	80, 90, 75, 70, 82, 78,	79	79	2,000,000,000	0.060
	2	82, 83, 66, 84, 77, 81	79			

108M suspension sufficient to give visible clouding, lysis followed. Furthermore, the phage could be transferred serially on 108M organisms so long as C lysin was incorporated in the medium. On the basis of these experimental results, the conclusion seems warranted that, *in vitro*, C lysin completely removes the capsule from both living and formalin-killed cultures of the type A *Klebsiella pneumoniae* strain tested.

Effect of C lysin on SSS. SSS was prepared from strain 108M as follows: Twenty-four hour tryptose glucose agar cultures of this strain were suspended in physiological salt solution and autoclaved for 15 minutes at 15 pounds pressure. The cells were then thrown down by centrifugation and discarded. The supernatant fluid was made slightly alkaline by the addition of N NaOH, sodium acetate was added to 2 per cent concentration, and the fluid was diluted with 3 volumes of 95 per cent ethyl alcohol. At this point a fairly voluminous precipitate formed. After 24 hours' storage in the refrigerator, the precipitate was collected on a fritted glass filter, washed with several small portions of alcohol, redissolved in water, and sterilized by filtration. This SSS solution, when tested
; a monospecific M antiserum, was precipitated to a titer of 1:10,000, but

failed to give a positive precipitin test with a strong S antiserum in a dilution of 1:10.

Treatment of this SSS preparation with C lysin UF caused no significant reduction in its precipitinogen activity. The results of an experiment of this nature are summarized in table 3. It may be seen from this table that SSS precipitinogen activity was the same after treatment with C lysin UF and with heat-inactivated C lysin UF.

Liberation of SSS from cells by C lysin. The inability of C lysin to destroy SSS has been confirmed by the following experiment: Four-ml volumes of a heavy suspension of 0.5 per cent formalinized 108M cells in broth were treated with 1-ml volumes of C lysin UF or heat-inactivated C lysin UF. The cells were then removed by centrifugation and the supernatants tested for SSS, using the pre-

TABLE 3

Effect of C lysin on precipitinogenicity of capsular polysaccharide

TUBE NO.	BROTH	SSS	C LYSIN	HEATED C LYSIN	PRECIPITINOGEN TITERS[*]	
					1:100	1:1,000
	ml	*ml*	*ml*	*ml*		
1	4	½	None	½	++++	+
2	4	½	½	None	+++	+

[*] After incubation 37 C/48 hr.

TABLE 4

Liberation of SSS from formalinized 108M cells by C lysin

TUBE NO.	108M SUS-PENSION	C LYSIN	HEATED C LYSIN	CELL VOL.[†]	PRECIPITINOGEN TITERS[*] OF SUPERNATANTS[†]		
					1:10	1:100	1:1,000
	ml	*ml*	*ml*				
1	4	1	None	0.007	++++	+++	—
2	4	None	1	0.017	+	—	—

[*] Monospecific 108M antiserum used.
[†] Incubation 37 C/4 hr followed by centrifugation in Hopkins tubes.

cipitin test. The results of this experiment are summarized in table 4. As can be seen from these data, treatment of the formalinized cells with C lysin resulted in the appearance of precipitable SSS in the medium.

Physicochemical Properties of C Lysin

Stability. These C lysin filtrates and ultrafiltrates are quite stable. Storage at refrigerator temperatures for periods of several months has resulted in no significant deterioration in C activity. The stability, at least for short time intervals, is relatively independent of pH. Exposure of filtrates to varying hydrogen ion concentrations from pH 9 to pH 5 for 4 hours at room temperature resulted in no appreciable loss of zone productivity. However, further increase in either acidity or alkalinity caused progressive loss of C activity.

Compared to phage MA8, C lysin is relatively heat-stable. However, the

lysin is readily inactivated by heat; 80 C for 10 minutes results in some loss of activity, and 85 C for 10 minutes results in complete inactivation.

Nondialyzability and precipitability. C lysin fails to diffuse through cellophane membranes; nor does dialysis result in any loss of activity. Prolonged dialysis does not result in precipitation of the active principle. However, C lysin is readily precipitated. Thirty per cent saturation with ammonium sulfate precipitates most of the activity, and 40 per cent saturation salts out all of it. The precipitate is readily soluble in water and no loss of activity occurs. Precipitation also occurs upon the addition of 2 volumes of acetone or of 5 volumes of 95 per cent ethyl alcohol. Exposure to the alcohol must not be prolonged as activity is destroyed by this reagent. Acetone seems to be less destructive.

C Lysin Specificity

The correlation of phage lytic activity with serological type specificity suggested that C lysin capsule-dissolving action might be specific for type A capsular

TABLE 5

Specificity of C lysin

STRAIN	TYPE C	LYSIN ACTIVITY
Klebsiella pneumoniae, Sc	A	+
" " , 16	A	+
" " , 103	A	+
" " , 145	A	+
" " , 2	B	−
" " , 3	C	−
Aerobacter aerogenes , K		−
" " ,C1P		−
Klebsiella pneumoniae, 108	A	+

+ = zone produced.
− = no zone produced.

material. To test this point C lysin UF was examined for zone-type clearing activity against 4 additional type A Friedländer strains, 1 type B *K. pneumoniae*, 1 type C *K. pneumoniae*, and 2 encapsulated *Aerobacter aerogenes* strains.

Young broth cultures of each of these bacteria were smear-streaked onto portions of the surface of an agar plate so as to give isolated solid patches of growth. Strain 108M was included as a control. The plate was incubated overnight at 37 C. All of the cultures developed as confluent, opaque, mucoid growth films. Loopfuls of C lysin UF were then spot-inoculated onto the surfaces of these bacterial films, and the plates were examined after a further incubation period of 20 hours at 37 C. The results of this experiment are summarized in table 5. As may be seen from this table, C lysin produced zone-type clearing in the growth films of the 5 type A cultures but produced no change in the growth of the other test cultures.

On the basis of these few test strains, C lysin activity would appear to be correlated with serological type specificity. The possibility of obtaining similar

lysins specific for types B and C *Klebsiella pneumoniae* cultures is under investigation in our laboratory.

DISCUSSION

C lysin is similar in all major aspects to the lysins described by Sertic and others. It is responsible for the gradually widening zones surrounding the phage plaques; it produces zone-type clearing in adult growth films of susceptible cultures; it may be separated from, and its activity is independent of, the phage; it is apparently nontoxic to cell growth and produces no permanent change in the culture; and it removes a constituent from the surface of the bacterial cells. C lysin dissolves the capsule from the *Klebsiella pneumoniae*, type A, cells, a fact clearly demonstrated by the loss of type specificity, the reduction in cell volume, and the appearance of SSS in the suspending fluid.

The engendered susceptibility of strain 108M, when grown in the presence of C lysin, to S phase phage and to S antibodies apparently is an analogous phenomenon explainable on the following purely physical basis: The intact capsule acts as a barrier preventing contact between the phage and the specific phage adsorption sites on the cells in the former case, and between the S antibodies and the specific somatic surface antigens in the latter case. Solution of the capsule by the lysin removes the barrier. On this basis, the developed sensitivity of the lysin-treated culture to the S phage and to the S antibodies is interpreted as indirect corroborative evidence that the lysin dissolves the capsule.

The results of these studies support the conclusion that C lysin is an enzyme. The factor is highly specific in its capsule-dissolving activity. It is either the disperse phase of a colloidal system or intimately associated with it, a view supported by the failure of the active principle to dialyze through cellophane. It is salted out by ammonium sulfate. It is soluble in water but insoluble in the fat solvents acetone and alcohol. It is inactivated by heat, strong acid, and alkali.

The mode of C lysin action against the capsule is obscure. As determined by chemical means, the capsule of *K. pneumoniae*, type A, is composed of a non-reducing, acidic polysaccharide containing glucuronic acid and yielding reducing sugars on hydrolysis (Goebel and Avery, 1927). Therefore, it seemed reasonable to suspect that the capsule-dissolving action of C lysin was the result of its poly-saccharide-hydrolyzing activity. However, since C lysin results in the liberation from the cells of SSS, and since it causes no reduction in the precipitinogen titer of our chemically prepared polysaccharide, C lysin would seem not to be a hydrolase.

Two ways in which the enzyme may act suggest themselves: The capsule surrounding these *Klebsiella* cells, as demonstrated by staining techniques, appears to be a well-defined morphological structure. If this structure is made up of long chain polysaccharide molecules, then these units must be linked by intermolecular forces; otherwise, no structural envelope could exist. It is possible, therefore, that the enzyme is a depolymerase which produces capsule solution by breaking the intermolecular linkage between SSS molecules.

The full antigenicity of the Friedländer capsular polysaccharide molecule *in*

situ, coupled with its haptenic antigenicity when in solution (Julianelle, 1926), suggests the possibility that SSS molecules may be, when on the cell, hooked with some other cellular constituent (nucleoprotein?), resulting in a complete antigen in which the polysaccharide portion is the determinate grouping. If this is true, then C lysin may split this molecular complex, liberating the SSS, and thus play in a specific manner the same role as do the nonspecific chemical methods of extracting the polysaccharide.

The correlation of lysin specificity and serological type specificity is in accord with the results of certain studies on the enzymic digestion of pneumococcus polysaccharides. Dubos and Avery (1931) isolated an enzyme from a sporulating bacterium which digested the capsule of type III pneumococci but failed to hydrolyze the capsular material of the other serological types tested. Enzymes specifically active against pneumococcus types III and VIII have been described by Sickles and Shaw (1933, 1935). Should C lysin specificity be further substantiated, the lysin may well serve as a useful practical tool in the identification of type A Friedländer cultures.

The occurrence of a capsule lysin in the phage lysates of this *Klebsiella pneumoniae* strain is of therapeutic interest because the factor has potential *in vivo* applicability. *Klebsiella pneumoniae* is a pathogen whose disease-producing ability is intimately associated with encapsulation.

Studies on the origin of C lysin, the nature of its action, and its effect on *Klebsiella pneumoniae* infections in laboratory animals are being carried on.

SUMMARY

Filtered phage lysates of a *Klebsiella pneumoniae*, type A, culture (strain 108M) contain a lysin that decapsulates both living and killed type A Friedländer cells. The activity of this capsule lysin ("C lysin") is independent of phage lytic action. Lysates treated with formalin or subjected to heat sufficient to inactivate the phage retain the capsule-dissolving powers. The C lysin may be separated from the phage particles by ultrafiltration through graded collodion membranes.

The phage-free lysin is nontoxic to the growth of the *Klebsiella* culture.

Two methods are described for the titration of the potency of various C lysin preparations using agar plate cultures.

Treatment of living or killed *Klebsiella pneumoniae* 108M cultures with C lysin results in a loss of serological type specificity and a marked reduction in cell volume. Injection of C-treated 108M cells into rabbits fails to elicit the production of type-specific antibodies. Treatment of living cultures with lysin results in an acquired susceptibility to a phage active only against the S, nonencapsulated form of this *Klebsiella* organism.

C lysin is highly specific; of the encapsulated cultures tested, only Friedländer type A strains are affected.

The mode of action of the lysin is unknown.   Chemically prepared SSS does not lose its haptenic properties as the result of treatment with C lysin, and SSS is liberated from these encapsulated bacterial cells upon treatment with the lysin.

The factor is relatively heat-labile (85 C for 10 minutes) and is rapidly inactivated by strong acid and alkali.   It is nondialyzable and is precipitated by ammonium sulfate, acetone, and alcohol.

Because of its specificity; heat, acid, and alkali lability; nondialyzability; and precipitability, it is concluded that C lysin is an enzyme.   The substrate is as yet unknown.

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NOTES

DETECTION OF FERMENTATIVE VARIANTS WITH TETRAZOLIUM[1]

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While trying to develop methods for finding dehydrogenase mutants, we found tetrazolium (triphenyl tetrazolium chloride) to be useful for detecting fermentative variants. This indicator has the advantage that the nonfermenting variants are stained a deep red whereas the fermenters and the background are neutral.

For example, cultures of *Escherichia coli* K-12 were spread on nutrient agar plates containing 1 per cent carbohydrate and 0.005 per cent tetrazolium. The plates were then irradiated with an ultraviolet lamp long enough to leave 100 to 500 colonies per plate after incubation. Red colonies or sectors were transferred to EMB agar to purify and verify them as mutants. Yields, ranging from 0.1 per cent to 0.01 per cent of the survivors, of mutants unable to ferment lactose, maltose, or glucose, respectively, have been obtained.

The response is undoubtedly due to the pH dependence of the biological reduction of tetrazolium to the highly colored, insoluble formazan. This usually accumulates in a single large granule near one pole of the cell.

Since *Salmonella* and *Shigella* cultures show a strong red reaction on lactose tetrazolium agar, this medium might be developed as an indicator for the enteric pathogens. However, it will be necessary to eliminate the reactions given by weak lactose fermenters (by controlling the pH and buffer capacity of the medium) and probably also to incorporate a selective inhibitor such as brilliant green.

MICROBIOLOGY OF SPRAY-DRIED, WHOLE-EGG POWDER

V. Aerobic Mesophilic Sporeforming Bacilli Isolated at 55 C[1]

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During the 14-month period, November 1, 1943, to January 1, 1945, taxonomic studies were made on the microbial population of egg powder samples as determined from glucose tryptone agar (Difco) plates incubated for 48 hours at 55 C (131 F) in a forced-draft incubator. This information was obtained simultaneously with microbiological findings for 5,725 samples of high-moisture (4 to 6 per cent), spray-dried, whole-egg powder manufactured in the United States for Lend Lease shipment (McFarlane *et al.*: Food Research, **12**, 311; Solowey *et al.*: Am. J. Pub. Health, **37**, 971; Sutton and McFarlane: Food Research, **12**, 474; Watson and McFarlane: Food Tech., **2**, 15). The investigation revealed that the organisms isolated at this temperature and suspected of being thermophiles were aerobic, mesophilic, sporeforming bacteria.

The 278 strains of bacteria selected for taxonomic study were from 600 isolations, and came from 177 samples of egg powder submitted by 32 dehydration plants located in 15 different states.

Morphological and biochemical findings revealed that 271 of the representative 278 strains were *Bacillus subtilis* and 7 were *Bacillus brevis*. These strains were all found to be capable of growth at 55 C (131 F) on slants of tryptone glucose extract agar immersed in a constant temperature bath for 48 hours.

The fact that all of the 278 representative cultures were aerobic, mesophilic, sporeforming bacilli is reasonably good evidence that most of the organisms responsible for the 55 C plate counts were of the same kind. The possibility, however, that thermophilic bacteria may be present in egg powder has not been eliminated. The ability of these species of aerobic, mesophilic, sporeforming bacilli to grow at temperatures ranging from 20 to 55 C is well recognized. It is possible that their presence in a given egg powder sample may be directly related to the quality of the shell eggs processed and the degree of plant sanitation practiced.

PROPERTIES OF SOME COLONIAL PHASE VARIANTS
OF CORYNEBACTERIUM RENALE[1]

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Five cultures, representing four different colony types, were chosen from stock cultures of *Corynebacterium renale* for study in order to determine their morphological, biochem cal, serological, and pathogenic relationships.

One culture that might be classified as "smooth" showed the weakest action in the fermentation of glucose and in the digestion of casein, had the least resistance to the bactericidal action of bovine plasma, and was the least pathogenic for rabbits. Two cultures that might be classified as "rough" showed the most resistance to the bactericidal action of bovine plasma and were the most pathogenic for rabbits. The other two cultures were intermediate in colonial morphology and pathogenicity.

The results of agglutination tests showed that there were antigenic differences and similarities between the different cultures, but there was little or no correlation with the morphological characteristics of the colonies.

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THE CONSEQUENCES OF MUTATION DURING THE GROWTH OF BIOCHEMICAL MUTANTS OF ESCHERICHIA COLI

I. The Pattern of Adaptation of Histidineless Cultures[1]

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A bacterial culture may contain a very large number of organisms. Consequently, even mutations occurring at very low rates per organism may not be uncommon. For this reason bacterial cultures are likely to be heterogeneous and their composition will depend upon mutation rates and selection pressure (Ryan, 1948). When mutations influence the growth characteristics of bacteria, the behavior of the whole culture may be drastically influenced. In the case of the so-called biochemical mutations, which affect the organisms' capacity to synthesize amino acids and growth factors, this influence is very evident. For example, by overgrowth during serial transfers in the presence of limiting concentrations of tryptophan, Wright and Skeggs (1945) have been able to select a strain of *Lactobacillus arabinosus* that does not require this substance. In the absence of tryptophan this new strain grows as fast and as far as does its tryptophan-dependent parent in the presence of tryptophan. Increasing concentrations of tryptophan, however, first depress and then allow this rapid growth of the tryptophan-independent mutant. Although the factors underlying this behavior are not yet understood in *L. arabinosus*, we have found a parallel situation in a histidineless mutant of *Escherichia coli*. This paper will present data that show that the depression of growth on intermediate concentrations of amino acid is the result of mutation and the consequent interaction between mutants and parents. The next two papers in this series will analyze the mechanism of this interaction (Ryan and Schneider, 1949a,b).

EXPERIMENTAL PROCEDURES

The strain of *E. coli* (148–334) used in these experiments was secured after X-radiation by Drs. J. O. Lampen and R. R. Roepke. It requires histidine for growth and was carried in stock culture on nutrient agar slants that were transferred every 2 months. Inocula were prepared by introducing with a needle a small amount of the culture (ca. 10^7 bacteria) into a 12-ml pyrex centrifuge tube containing about 5 ml of chemically defined medium supplemented with 25 μg of Eastman L-histidine monohydrochloride·H_2O per ml. Growth was allowed to occur for about 12 hours at 37 C at the end of which time the cotton plug was fastened with scotch tape and the bacteria in the tube were centrifuged to the bottom. The supernatant was then decanted and the bacteria resuspended in

5 ml of 0.9 per cent saline. The process was repeated and after the second suspension the bacteria were said to be washed once. All dilutions were made in saline. At inoculation, one drop (0.05 ml), containing a total of about 2×10^7 bacteria, was introduced into 10 ml of synthetic medium (see Ryan and Schneider, 1948) containing the various experimental supplements.

Growth was allowed to occur in unshaken 16-by-150-mm pyrex test tubes at 37 C and was followed as optical turbidity by means of a Klett-Summerson colorimeter fitted with a no. 59 filter. The pyrex test tubes fit snugly into the adapter, whose opening was ground somewhat larger to receive them, and were calibrated for their own absorption when filled with water. The colorimeter was calibrated in terms of the number of bacteria (the histidineless [h−] and its histidine-independent [h+] mutant were the same in this regard irrespective of the conditions of growth) by plotting the concentration of bacteria against the instrument reading. The curves arose from the origin of ordinate and abscissa and were linear for about 100 colorimeter units. Over this range each unit represents about 5×10^6 bacteria per ml. Thereafter the calibration curve deviated with constantly decreasing slope from a straight line. At the level of 150 colorimeter units the reading actually represented 5 per cent more organisms than a continuing linear relationship would predict. At 200 the deviation was 10 per cent, and at 400, 16 per cent. Since only rarely did the density of experimental tubes exceed 150, growth is expressed, in this and in the following papers, as turbidity values without conversion to bacterial numbers. The viable number (determined by plating) and the total number (determined with a hemocytometer) were equal in cultures 24 hours old. For the histidineless strain grown on an optimal histidine concentration there were 48 and 47×10^7 organisms per ml respectively; for the histidine-independent, 56 and 48×10^7.

When cultures were to be characterized in terms of the two types of organisms they contained, plating procedures were used that have been described elsewhere (Ryan and Schneider, 1948). In principle the method involved plating into minimal agar where the h+ bacteria developed into colonies. Subsequent layering with histidine allowed the development of new colonies from the h− bacteria present.

RESULTS

Mutation. When an inoculum of histidineless (h−) bacteria, prepared as described above, is introduced into chemically defined medium containing 25 μg of histidine per ml, growth is complete within 12 hours. When, however, a similar inoculum is introduced into chemically defined medium without histidine (minimal medium), no growth is visible at 12 hours, but, depending upon conditions, occurs after some 24 hours. This growth in the absence of histidine is called adaptation because the culture has developed the ability to grow on medium which previously did not support growth. When an inoculum is prepared from such an adapted culture and introduced into fresh minimal medium, growth occurs within 12 hours without the long lag period exhibited by the parent strain. From adapted cultures it is possible to secure by plating and colony isolation pure strains of bacteria that are able to grow normally in the absence of histidine.

These histidine-independent strains (h+) synthesize their own histidine as determined by the bioassay of their hydrolyzates with h− bacteria. Histidine independence is inherited indefinitely in a stable fashion. Among 15,783 h+ bacteria identified from h+ cultures grown in the presence of histidine, no h− bacteria were found. On the other hand, h+ organisms are found in all h− cultures with a frequency of about 10^{-7}. They even appear in h− cultures established from single h− bacteria. For this reason it is certain that h+ bacteria arise from h− by the mutation designated h− → h+ (Ryan, 1948).

An inoculum of 0.05 ml of an h− culture contains an average of about 3×10^7 h− bacteria and about 3 h+ bacteria. A 10^{-7} dilution of an h− culture contains no h+ bacteria and about 50 h− organisms per ml. Yet when 1-ml inocula of such dilutions of washed h− cultures are introduced into minimal medium devoid of added histidine, adaptation occurs due to the appearance and growth of h+ bacteria. When, however, such 10^{-7} dilutions are introduced into minimal medium that is devoid of histidine and from which asparagine (the only organic substance except glucose which is normally present in minimal medium) has been omitted, adaptation never occurs. This discrepancy is due to the fact that the asparagine preparation used contains about 0.003 per cent histidine (Ryan and Schneider, 1948). This small amount of histidine allows for the slow growth of enough h− bacteria to make mutation to the h+ condition probable. It is the growth of these newly arisen h+ bacteria that constitutes adaptation. In the absence of the asparagine preparation (and hence in the complete absence of histidine) the few h− organisms in a 10^{-7} inoculum do not grow at all, and mutation with consequent adaptation does not take place; h+ organisms (and hence a larger inoculum) must be introduced for adaptation to occur under these conditions. Otherwise the absence of asparagine results only in a slight lengthening of the lag period of h− and h+ bacteria; the logarithmic rates of growth and the final yields of bacteria are the same in the presence and in the absence of asparagine.

The pattern of adaptation. When very small amounts of histidine, which would allow only a limited growth of h− bacteria, are added to minimal medium without asparagine, 10^{-7} inocula are enabled to adapt. However, the extent to which the mutant h+ bacteria derived from them grow, and consequently the extent to which adaptation occurs, is a function of the concentration of histidine present. Figure 1 shows the adaptation achieved by a 10^{-7} inoculum of an h− culture in a medium devoid of asparagine but containing increasing concentrations of histidine. No adaptation occurred in the absence of histidine, for no h+ cells were introduced with the inoculum nor were they produced in the absence of growth of the h− bacteria that did form the inoculum. On 0.1 μg of histidine per ml, adaptation did occur owing to the formation of h+ bacteria produced during the initial multiplication of the h− organisms. However, adaptation becomes progressively less extensive as the histidine concentration is increased. At and above the level of 1 μg per ml, adaptation does not occur even after a period of 2 weeks. This phenomenon, portrayed by the dip in the adaptation curve, has been the subject of extensive investigation in our laboratory.

When asparagine is present in the medium, adaptation in the absence of added
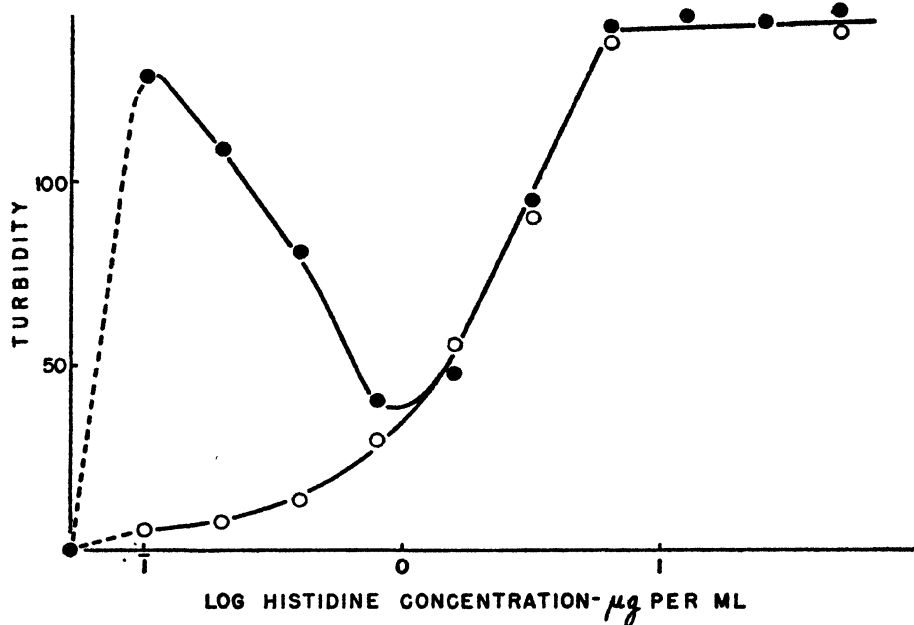
Figure 1. The adaptation of cultures of h— bacteria grown on different concentrations o histidine in medium devoid of asparagine. The inoculum in this experiment consisted o the ca. 50 h— bacteria present in a 10^{-7} dilution. There were no h+ bacteria in the inoculum. The lower curve shows the stationary level of growth achieved after 46 hours, before adaptation occurred. The upper curve shows the stationary level of growth achieved after 76 hours, when adaptation was complete.
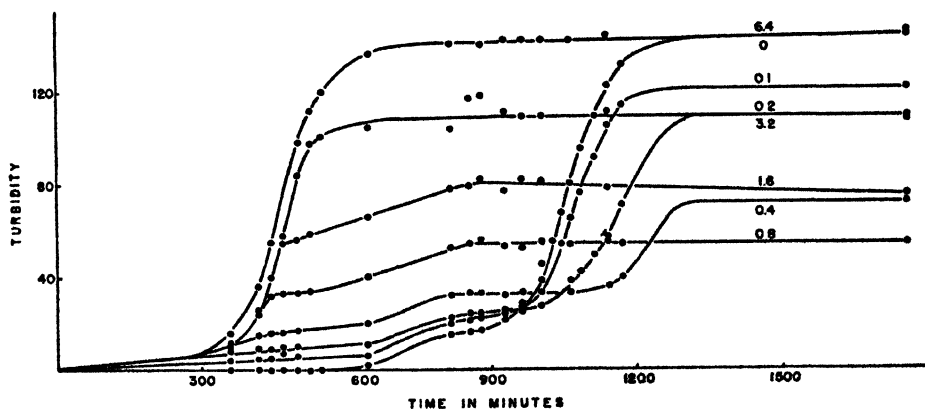
Figure 2. The time-course of adaptation of h— cultures grown on different concentrations of histidine. The numbers refer to the concentrations of histidine in µg per ml. This experiment was chosen for publication because of its completeness. The kink in the pre-adaptive curve was peculiar to this experiment and was not found in numerous repetitions.

histidine does take place and growth of the adapted bacteria is equal in amount to that achieved by h— bacteria grown in the presence of an optimal amount of

histidine.   This is shown in figure 2, which also portrays the time course of adaptation on different concentrations of histidine.   Once again, in the presence of asparagine, the amount of adaptation is inversely proportional to the histidine concentration.   Even after 2 weeks there is no change in the final level of growth shown in this figure.   The relation between adaptation and histidine concentration is clearly shown in figure 3, which portrays sections through figure 2 at 11 and 29 hours.   Adaptation does not occur at levels above 1 μg per ml.   When adaptation does occur, it is the consequence of the production of h+ mutants in
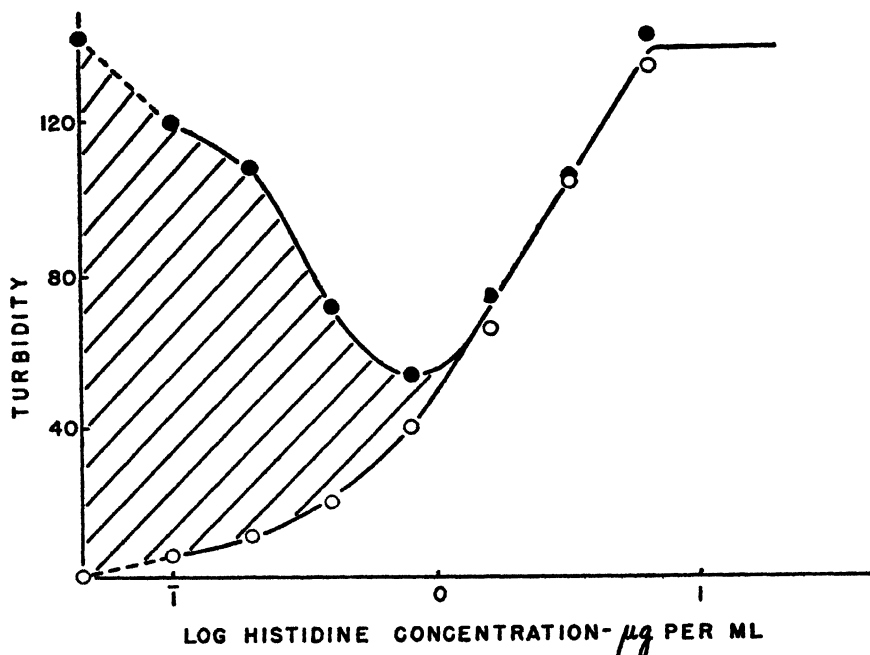
Figure 3. The relation of the amount of growth before and after adaptation to histidine concentration.   The lower curve is for growth at 11 hours and the upper curve for growth at 29 hours.   The shaded area indicates the amount of adaptation.   These data are the same as those shown in figure 2.

the experimental tubes.   These mutants were derived from the h— bacteria that multiplied owing to the traces of histidine introduced with the asparagine. In addition, the 0.05 ml of washed, undiluted h— cultures used as inocula contained an average of 3 h+ bacteria that were also involved in adaptation.   Unless otherwise mentioned, the experiments reported in this paper were performed with a medium containing asparagine.

The h+ bacteria when grown by themselves have growth characteristics that are similar to those of h— bacteria grown in the presence of optimal histidine (figure 4 and table 1).   Table 2 shows how, on the contrary, when h+ and h— bacteria are together, as is the case during adaptation, the behavior of the mixed culture is drastically different.

The cause of the adaptive pattern. When cultures of h— bacteria that had adapted on various histidine concentrations were plated out and the proportion of h— and h+ organisms was determined, the data shown in figure 5 were secured. As adaptation is more complete, there are progressively smaller percentages (and numbers) of h— bacteria. Those cultures that do not adapt

TABLE 1

Effect of histidine concentration on the growth characteristics of h+ bacteria and of h— bacteria before adaptation

BACTERIA	HISTIDINELESS			HISTIDINE-INDEPENDENT		
Histidine concentration; µg per ml	Time in minutes required to produce a turbidity of 40	Rate during linear growth $\frac{\text{turbidity}}{\text{time}}$	Final turbidity before adaptation	Time in minutes required to produce a turbidity of 40	Rate during linear growth $\frac{\text{turbidity}}{\text{time}}$	Final turbidity
0	—	0	10	345	0.54	145
0.1	—	0.01	14	365	0.55	140
0.2	—	0.02	20	360	0.53	141
0.4	—	0.03	30	345	0.53	141
0.8	540	0.06	50	360	0.56	146
1.6	420	0.31	72	380	0.53	141
3.2	380	0.56	108	360	0.53	142
6.4	365	0.55	143	350	0.54	142
12.8	335	0.49	144	355	0.54	138
25.6	320	0.52	142	355	0.55	145
51.2	315	0.46	144	355	0.50	145
102.4	315	0.50	141	360	0.49	142

TABLE 2

Effect of histidine concentration on the growth characteristics of an h— culture during adaptation

HISTIDINE CONCENTRATION, µg PER ML	TIME IN MINUTES REQUIRED TO PRODUCE A TURBIDITY OF 40	RATE DURING LINEAR ADAPTIVE GROWTH $\frac{\text{TURBIDITY}}{\text{TIME}}$	FINAL TURBIDITY AFTER ADAPTATION
0	1,000	0.65	151
0.1	1,010	0.55	127
0.2	1,080	0.44	110
0.4	1,220	Curved	77

possess more than 99 per cent h— bacteria. The remarkable circumstance is that in the presence of 0.4 and 0.8 µg histidine per ml, where there is very little adaptation, there are appreciable numbers of h+ bacteria. These bacteria are alive despite their failure to multiply. An inoculum from a culture of h— bacteria which had become adapted in the presence of 0.4 µg histidine per ml and which contained 72 per cent h— bacteria was inoculated into fresh medium containing 0.4 µg histidine per ml. The new growth consisted of 7 per cent h— bacteria, a proportion in accord with results secured with deliberately mixed
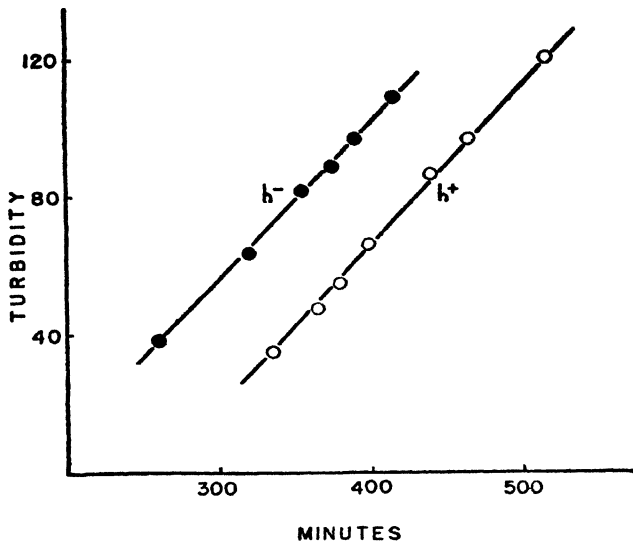
Figure 4. The linear rates of growth of h— and h+ bacteria in the presence of 25.6 μg histidine per ml. Both strains were grown in unshaken cultures.
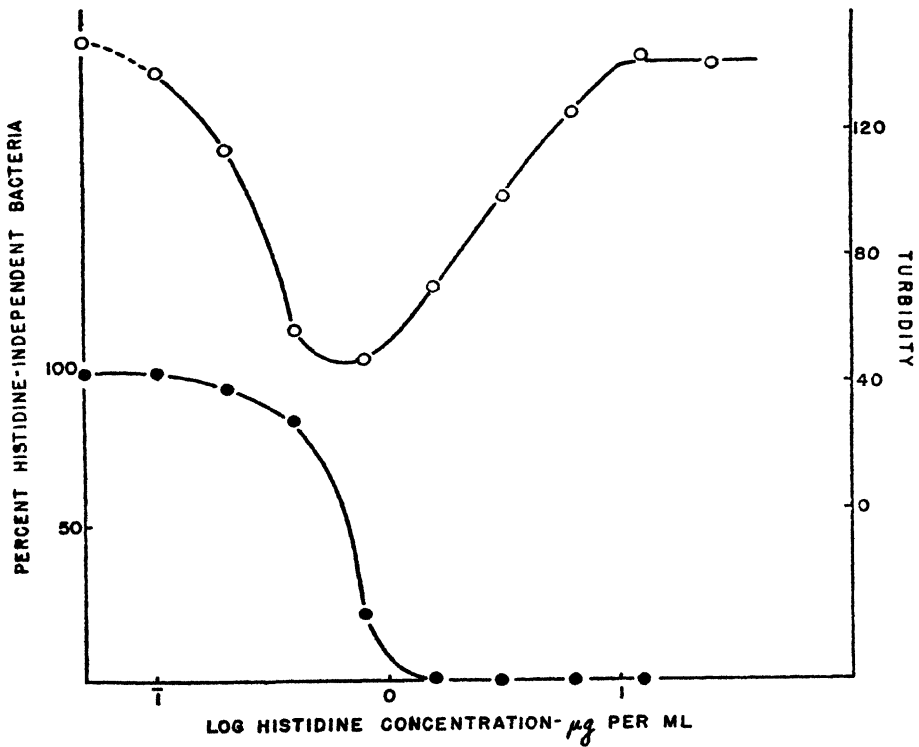
Figure 5. The percentage of h+ bacteria in cultures allowed to adapt in different concentrations of histidine. The upper curve shows the amount of growth achieved after adaptation (28 hours). The lower curve shows the proportion of h+ organisms in the various adapted cultures.
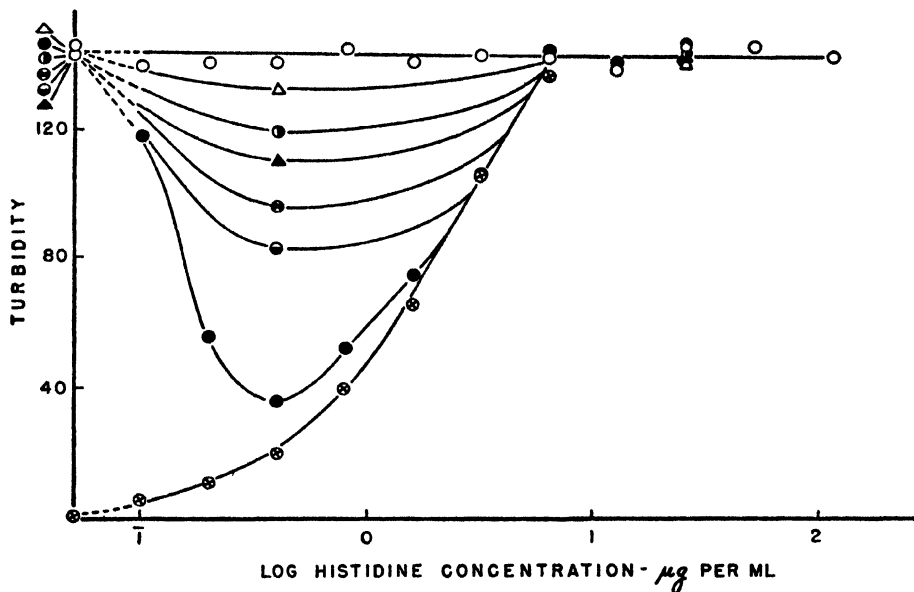
Figure 6. The adaptation of cultures started with inocula consisting of various proportions of h+ and h− bacteria. The lowermost curve shows the level of growth after 12 hours (before adaptation) of a culture inoculated with a mixture containing 99.9999 per cent h− bacteria. Above this in order are curves for the adaptation of inocula containing 99.9999, 99.999, 99.99, 99.9, 99, 54.5, and 0 per cent h− bacteria. The inoculum containing 0 per cent h− bacteria was secured from a pure h+ culture.
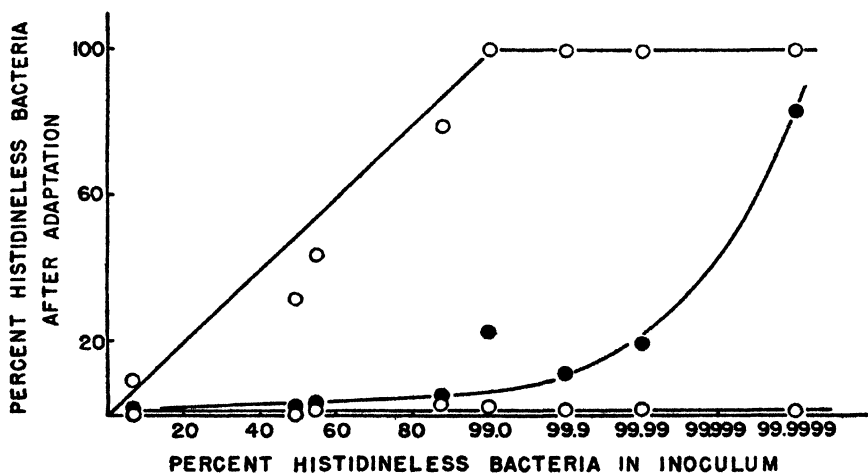
Figure 7. The change in proportion of h− bacteria after adaptation. The upper curve is for growth in the presence of 25.6 μg histidine per ml, the next for 0.4 μg, and the bottom curve for no histidine. All data were secured 24 hours after inoculation. Note the change in the scale of the abcissa.

inocula (see below). The problem is, then, to discover what prevents the h+ bacteria in partially adapted cultures from continuing growth and adaptation.

The depression in the adaptation curve of h— bacteria is similar to that obtained by growing a heterocaryon of *Neurospora* containing leucineless and leucine-independent nuclei on different concentrations of leucine (Ryan, 1946, figure 5). In the latter case it was shown that in the presence of leucine the leucineless nuclei had a selective advantage over their leucine-independent neighbors. Similarly, we might assume that the h+ bacteria are prevented from growing by the presence of too many h— bacteria. If so, it should be possible to eliminate the depression of growth which occurs on intermediate concentrations of histidine by decreasing the percentage of h— organisms in the inoculum.

Actually, this is possible, and, as figure 6 shows, progressive decreases in the proportion of h— bacteria in the inoculum result in progressively less depression of growth. Eventually when there are no h— bacteria in the inoculum, growth is independent of histidine. This is true of a pure culture of h+ bacteria. When, however, h+ bacteria are mixed with h— bacteria, their growth is depressed. We must conclude that the depression is brought about by h— bacteria.

During adaptation, the proportion of h— and h+ bacteria changes from what it was in the inoculum. Figure 7 shows this shift on three different concentrations of histidine. In the presence of an optimal concentration there appears to be no selective advantage in favor of h+ bacteria. In the absence of histidine there is a very strong selection for h+ bacteria which, regardless of their proportion in the inoculum, make up the bulk of the adapted population. On a limiting concentration of histidine the h+ bacteria are selected more strongly when there are large numbers of them present in the inoculum. This is additional evidence that large numbers of h— bacteria inhibit the growth of h+ bacteria.

DISCUSSION

These data show that the amount of adaptive growth of an h— culture is a function of the histidine concentration. The more h— bacteria, the less adaptation. Further, this depression of adaptation is brought about in some way *by* the h— bacteria. The following papers in this series will consider the mechanism of this influence (Ryan and Schneider 1949*a*,*b*). They will also show that this inhibition of the growth of h+ organisms is a phenomenon that occurs with its greatest effect on intermediate, suboptimal concentrations of histidine. Moreover, competition between h— and h+ bacteria is in favor of the latter in the absence of histidine but does not occur in the presence of optimal concentrations (Ryan, 1948). Figure 7 of the present paper shows that h+ bacteria are selected in the presence of a limiting concentration of histidine (0.4 μg per ml). But even though selection is in favor of h+ bacteria, they are eventually inhibited by the h— bacteria present. This is a unique property of h— bacteria grown on intermediate suboptimal concentrations of histidine. On very low concentrations adaptation does occur. On optimal concentrations, where there is little if any inhibition of h+ by h— bacteria, adaptation does not occur because the h— bacteria have undergone all the growth the medium will support.

SUMMARY

During the growth of populations of histidineless *Escherichia coli*, mutations occur which make some of the bacteria independent of an external supply of his-

tidine. These histidine-independent mutants grow in the absence of added histidine, and such growth from an inoculum of histidineless bacteria is termed adaptation.

The extent to which adaptation occurs is inversely proportional to the histidine content of the medium. On high concentrations adaptation does not take place at all. As a result there is a dip in the curve describing adaptive growth as a function of histidine concentration.

This dip can be progressively eliminated by adding histidine-independent bacteria, which artificially decreases the proportion of histidineless bacteria in the inoculum. Pure histidine-independent cultures show no dip in the curve, for their growth is not influenced by histidine in the medium.

The depression of adaptation is a function of the number of histidineless bacteria present. As the histidine concentration increases, the number of histidineless organisms whose growth is supported increases, and the adaptative growth of histidine-independent mutants decreases. The latter are in some way prevented from growing by histidineless bacteria grown on suboptimal concentrations of histidine.

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ENZYMATIC STUDIES ON THE MECHANISM OF THE RESISTANCE OF PNEUMOCOCCUS TO DRUGS

I. Studies of the Dehydrogenase Activities and Interrelationships of Pneumococci Susceptible and Resistant to Acriflavine, Atabrine, Optochin, Propamidine, and Sulfonamides[1]

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Despite the numerous studies for over fifty years on the resistance manifested by microorganisms to various drugs, a satisfactory concept concerning the biochemical mechanism of resistance development has not as yet evolved. The many observations that suggest a correlation between flavoprotein activity and the acquisition of resistance to certain drugs have been analyzed by one of us (Sevag, 1946). The studies here presented were oriented toward an experimental elucidation of these observations.

The pneumococcus was used in these investigations sinceit represents a pathogenic organism whose respiratory activities are principally dependent on the flavoprotein enzymes (Avery and Neill, 1924; Sevag, 1933; Sevag and Maiweg, 1934; Sevag and Shelburne, 1942). Strains of three different serological types (I, II, and III) were made resistant independently to each of five different drugs by subjecting the organisms to increasing concentrations of the drugs *in vitro*. The resistant strains and their susceptible parent strains were studied for their dehydrogenase activities using methylene blue as the hydrogen acceptor to measure the flavoprotein activity. This is valid on the basis that neither methylene blue, oxygen per se, nor oxygen with activation by the cytochrome oxidase system is capable of oxidizing the dihydropyridine cozymases (I and II or DPN and TPN). It is believed that the flavoprotein is an enzyme whose specific substrates are the reduced cozymases, the hydrogen, originally obtained from the substrates oxidized, being transferred from the reduced pyridine ring of the cozymases to the iso-alloxazine ring of the flavoprotein. The hydrogen of the reduced flavoprotein may then under appropriate conditions be transferred to a hydrogen acceptor in the system, in the present case to methylene blue.

The agents investigated were acriflavine (3,6-diamino-N-methyl acridinium dihydrochloride—National Aniline and Chemical Co.), atabrine dihydrochloride (Winthrop no. AY-392), propamidine (4,4'-diamidino-diphenoxypropane—

Merck no. 3R-8008)[3], optochin (ethyl-dihydrocupreine hydrochloride—Merck), and finally sulfonamides.[4] The first two represent typical acridine dyes that bear configurational similarity to the alloxazine nucleus of the flavoprotein coenzymes. The action of several acridines has been attributed to their possible interference with the yellow enzyme systems (McIlwain, 1941; Haas, 1944; Wright and Sabine, 1944; Madinaveitia, 1946; Hellerman, Lindsay, and Bovarnick, 1946; Michaelis, 1947). Propamidine represents a diamidine whose action appears to be similar to certain acridines in spite of dissimilarity in structure. This similarity is based on the observations (a) that there exists a common antagonism of the antibacterial action of propamidine and atabrine by certain polyamines (Silverman and Evans, 1943; Snell, 1944) and by riboflavin (Madinaveitia, 1946); and (b) that a striking development of cross resistance occurs between propamidine and some acridines (McIntosh and Selbie, 1943). Optochin, a quinoline derivative, is a specific pneumococcidal agent that exercises strong inhibition on the pneumococcal dehydrogenases (Schnabel, 1920). Its structure suggests only a vague similarity to the acridines and alloxazine. Since sulfapyridine-resistant pneumococci were reported to have a reduced ability to form hydrogen peroxide and decreased dehydrogenase activity (MacLeod, 1939), sulfonamides were included for investigation. Sulfapyridine and sulfathiazole were used in the experiments to follow.

EXPERIMENTAL PROCEDURES AND RESULTS

Development of Resistance

The organisms used were *Diplococcus pneumoniae*, type I (P27), type II (P28), and type III (P29). These were obtained from Dr. H. E. Morton. Stock cultures were maintained in blood broth (2 per cent horse blood in beef extract broth containing Parke, Davis peptone). Assays for drug sensitivity and the development of resistance were made in the semisynthetic medium (Adams and Roe, 1945) containing "casamino acids" (Difco). Twofold serial dilutions of the drugs were made in 5 ml of this medium and inoculated with 0.1 ml of an 18- to 24-hour culture in the same medium. For sulfonamide assay the inoculum was diluted to 1:1,000. Resistance was developed by transferring 0.1 ml of the 24-hour culture growing in the highest concentration of the drug to new series containing the same and higher concentrations. Such transfers were made daily with intermediate subculture to blood broth for stock purposes. Checks for the purity of cultures were made on blood agar plates (5 per cent horse blood in the tryptone glucose yeast extract medium of Morton and Engley, 1945, to which agar was added). A drug-free susceptible control was maintained similarly to the resistant strains but in the total absence of the drugs. Occasional mouse virulence tests were performed by intraperitoneal inoculation.

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[3] We are indebted to Dr. D. F. Robertson from Merck and Company for the supply of propamidine.

[4] On the basis of observations from Ehrlich's laboratory that atoxyl (sodium salt of *p*-aminophenylarsenic acid) has an action on trypanosomes similar to that of the acridines, atoxyl was tried. It was found that pneumococci were naturally resistant to this compound, surviving a 1:200 concentration.

The following data represent the experiences encountered in the development of resistance to the various drugs. Data are given as to (a) the degree of resistance obtained; (b) the number of transfers for development of maximal resistance; (c) the stability of resistance once obtained; and (d) the production of phase variation in certain instances. Mouse virulence was determined in at least one representative resistant strain for each drug. In all cases the characteristics of the mucoid form persisted, such as gross appearance of the colonies and mouse virulence. Type specificity, as determined by slide agglutination, and the Quellung reaction with specific immune serum remained unaltered for all resistant mucoid strains.

Acridines. The development of resistance in both acriflavine and atabrine followed a similar pattern. Many transfers in the respective drugs were required for the development of maximal resistance. In the case of acriflavine, the organisms were originally susceptible to a 1:800,000 to 1:1,600,000 concentration of the drug. After approximately 30 transfers they could grow in concentrations of 1:50,000 to 1:100,000. With atabrine, the initial inhibitory concentrations of 1:400,000 to 1:800,000 were raised to 1:25,000 to 1:50,000. Atabrine and acriflavine resistance was relatively unstable, tending toward a decrease in resistance in drug-free media. Before each experiment, therefore, it was necessary to transfer these organisms several times in the presence of the drug in order to maintain maximal resistance. With one exception, the mucoid characteristics of the organisms remained constant throughout exposure. A rough[*] resistant form of type III developed in atabrine. Upon repetition, a type III resistant mucoid form was obtained.

Propamidine. Propamidine presented no difficulties in the development of resistance. Maximal resistance could be obtained in as few as five transfers. Moreover, mucoid characteristics and the degree of resistance obtained remained stable throughout. Organisms that were originally inhibited by 1:320,000 (type I) and 1:160,000 (types II and III) were able to grow in 1:5,000 (type I) after 7 transfers, in 1:10,000 (type II) after 13 transfers, and in 1:5,000 (type III) after 5 transfers. Resistance remained stable during storage in drug-free media.

Optochin. Like propamidine, the development of resistance in optochin was rapid with maximal resistance, being obtained in as few as 4 transfers. Types I and III, which were inhibited by a 1:320,000 concentration of the drug, were able to grow in 1:40,000 after 4 and 10 transfers, respectively; type II, which was inhibited by a concentration of 1:800,000, could grow in 1:40,000 after 5 transfers. These were mucoid strains. However, the experiences of Amzel (1927) and Lewy (1925) were shared by us. During cultivation in the presence of optochin, rough variants appeared that lacked sensitivity to the drug. This appeared to be nonspecific in that rough variants of normal strains that had never been exposed to optochin were less sensitive to the action of the drugs than the original mucoid forms. The resistant mucoid strains were obtained by colony separation on blood agar plates as well as from alternate blood broth subcultures during

[*] The classification of the phase variants indicated by the terms mucoid, smooth, and rough, used here, is that of Dawson (1934).

transfers through the drug. A type II resistant rough form was maintained through drug transfer until maximal resistance developed, and was stocked for analysis. In all cases, the resistance, once obtained, remained unchanged even after subculture in drug-free media.

Sulfonamides. With sulfonamides, the development of resistance was slow and difficult. The organisms were originally subjected to sulfapyridine (SP), but because higher concentrations were needed than the low solubility of SP permits, transfers were continued in sulfathiazole (ST). After a total of 47 transfers, type I, which was originally inhibited by 1:32,000 (ST), was able to survive a 1:4,000 concentration; types II and III, originally inhibited by 1:64,000 (ST), became resistant to a concentration of 1:1,600.

Type I remained mucoid throughout. Types II and III, however, lost their typical mucoid characteristics. They appeared to be an intermediate phase between mucoid and rough, characterized by a loss of virulence for mice, diminished bile solubility, and autoagglutinability. They could not be identified as true rough forms on the basis of colony morphology. The agar plate colonies were lenticular, even, glistening, larger and more watery in appearance that the typical rough organisms. Resistance stability in the absence of these drugs was variable between strains, but the strains at the most showed only slight decrease in resistance.

A mucoid type II pair of parent strains, susceptible and resistant to SP, were made available to us through the courtesy of Dr. L. H. Schmidt of Christ Hospital, Cincinnati, Ohio (Schmidt, Sesler, and Dettwiler, 1942).

Cross Resistance

The resistant mucoid organisms were examined for susceptibility to the drugs to which they had not been habituated. Titrations were made by the usual procedure of the action of the various drugs on the resistant organisms in comparison with their action on the parent organisms. The optochin- and sulfonamide-resistant organisms were resistant only to the drug to which they had been exposed. The sulfonamide-resistant organisms were resistant to both sulfathiazole and sulfapyridine. In a few isolated instances the optochin-resistant organisms did show a twofold increase of resistance to sulfathiazole. The organisms which were exposed to atabrine, acriflavine, and propamidine were as susceptible to sulfathiazole and optochin as the parent culture which was not exposed to these drugs. The organisms which were made resistant to propamidine or acridines show a remarkable degree of cross resistance between acriflavine and propamidine. The results (table 1) are given in figures representing the times of increase in resistance over that of the original unmodified parent strain. Thus, it can be seen that organisms made resistant to propamidine are equally resistant to acriflavine though they were never in contact with the latter, and, conversely, organisms whose resistance was developed to acriflavine are equally resistant to propamidine. Atabrine shows some cross resistance with the others, but of a much smaller degree. It is interesting that the cross resistance is less evident between the two structurally similar acridines than between one of the acridines and a

structurally unrelated diamidine. McIntosh and Selbie (1943) have shown similar cross-resistant relationships between propamidine and some acridines and the staphylococci. Along with our observations on cross resistance and other previously mentioned data, it may appear that propamidine and some acridines possess a similar mode of action despite dissimilar structures. This will be more fully investigated and discussed in a later presentation.

Dehydrogenase Activity

Methods. The organisms were grown in a medium consisting of 1 per cent peptone (Parke, Davis), 1 per cent sodium acid phosphate, 0.3 per cent beef

TABLE 1

Cross resistance to growth inhibition among pneumococci resistant to acriflavine, atabrine, and propamidine

TYPE	ORGANISMS MADE RESISTANT TO	INCREASE (FOLD) IN RESISTANCE OVER PARENT SUSCEPTIBLE ORGANISM[*] IN PRESENCE OF		
		Acriflavine	Atabrine	Propamidine
I	Acriflavine	16	2–4	8–32
	Atabrine	4	16–32	4
	Propamidine	16	4	16–32
II	Acriflavine	8	2	4–8
	Atabrine	1	16	2
	Propamidine	2–4	2–4	8
III	Acriflavine	16	2	8
	Atabrine	1–2	16	2
	Propamidine	8	2	16

[*] Increase (fold) in resistance $= \dfrac{\text{Minimal inhibitory concentration for resistant organisms}}{\text{Minimal inhibitory concentration for parent susceptible}}$.

extract, and 0.5 per cent glucose. The inoculum added to 150 ml of medium consisted of 0.4 ml of blood broth culture which had grown for 6 to 8 hours. A solution of water-clear catalase (0.2 ml) prepared from rabbit erythrocytes (Sevag and Maiweg, 1936) was added to each bottle just prior to inoculation to allow growth free from the effect of the hydrogen peroxide formed. After 10 to 16 hours' incubation at 37 C the organisms were centrifuged, washed twice with M/20 phosphate buffer (pH 7.6), and resuspended in the same buffer. The weight of the organisms in a given suspension was adjusted by means of turbidimetric measurement with a photoelectric colorimeter to give uniform suspensions containing 1 mg of cells per ml (3 to 4 billion cocci). Fresh cultures were used for daily experiments.

Dehydrogenase activity was measured by means of the conventional Thunberg technique. The test systems consisted of 1 ml of pneumococcal suspension, 0.5

ml of methylene blue (0.002 M in M/20 phosphate buffer at pH 7.6), and phosphate buffer to give a total of 3.5 ml in the tube; 0.5 ml of substrate solution in phosphate buffer was put into the side arm. The tubes were attached to a manifold and evacuated for 3 minutes with a high vacuum pump. In this manner a uniform vacuum was produced in all the systems tested. After warming for 10 minutes in a 37 C water bath, the contents of the tube and side arm were mixed. The time required for 100 per cent reduction of the methylene blue was recorded in minutes (t_r).

Discussion of results. Glucose, glycerol, sodium lactate, fructose-1,6-diphosphate (HDP), and ethyl alcohol as substrates could readily be activated by the normal organisms. Under the conditions of our experiments, maleic, fumaric, succinic, and pyruvic acids could not be activated to serve as hydrogen donators. The methylene-blue-reducing activity of a system was dependent on the number of organisms and, with the exception of glucose, on the concentration of substrate.[6] In a given system the activities of the organisms harvested on different days were found to be variable. Consequently, all comparisons were made with experiments that were run simultaneously. The values given are based on averages of at least two, and as many as six, separate experiments.

Normal dehydrogenase activities of drug-susceptible pneumococci. Glucose was the most active of the substrates, giving a t_r of 30 minutes or less with 1 mg of organisms. The final concentration of glucose used throughout was 0.001 M. Glucose activity in type III was, in general, less than in the other types. Glycerol was less active as substrate, requiring about 3 times more organisms. Under these conditions the methylene blue reduction period with 0.1 M glycerol was similar to that obtained with 0.001 M glucose and 1 mg of organisms. Of the substrates used, lactate was the least active, requiring for 0.1 M concentration more than an hour for the complete reduction of methylene blue with 3 mg of type I organisms. However, in the type II organism the lactate dehydrogenase activity approximated that of glycerol and was at least twice as active as in the other types. The activity of organisms with hexose diphosphate (0.005 M) and ethanol (0.1 M) approached the activity shown with 0.001 M of glucose. However, further decrease in concentration of these substrates resulted in decreased activity. More than 3 mg of organisms could not be used because of the increase in endogenous activity. With 1 and 2 mg of organisms the endogenous activity was nil, requiring more than 6 hours for the reduction of methylene blue. With 3 mg it was more variable, but usually required more than 3 hours for the reduction of methylene blue.

Comparison of dehydrogenase activities of susceptible and resistant mucoid organisms. The degree of resistance, as shown in table 2 (column 2), is expressed as a ratio of the minimal concentration of drug required to inhibit the resistant organism to the minimal concentration required to inhibit the susceptible organism. It represents the number of times resistance increased.

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[6] With glucose as substrate, no difference in activity could be demonstrated in the range of 0.1 to 0.0001 M. Twofold variations in concentrations of the other substrates produced marked difference in activity.

ORGANISM	INCREASE (FOLD) IN RESISTANCE	METHYLENE BLUE REDUCTION TIME (MINUTES)			
		Glucose (0.001 M)	Glycerol (0.1 M)	Lactate (0.1 M)	Endogenous (no substrate)
Acriflavine					
Type I					
Susceptible...........	—	14 (2)*	32 (3)	67 (3)	200 (3)
Resistant............	20	50	26	139	>250
Type II					
Susceptible..... ...	—	16 (1)	40 (3)	32 (3)	143 (3)
Resistant	8–16	26	45	34	>200
Type III					
Susceptible......... ..	—	31 (1)	53 (3)	71 (3)	>250 (3)
Resistant.............	8–16	32	46	72	>250
Atabrine					
Type I					
Susceptible...... ..	—	14 (1)	28 (3)	89 (3)	>200 (3)
Resistant	16–32	230	33	127	>200
Type II					
Susceptible..	—	15 (1)	34 (3)	36 (3)	>200 (3)
Resistant..... ..	16–32	>250	44	57	>200
Type III					
Susceptible	—	31 (1)	57 (3)	74 (3)	>250 (3)
Resistant	16–32	150	44	136	>250
Propamidine					
Type I					
Susceptible...... ...	—	15 (1)	30 (3)	71 (3)	>250 (3)
Resistant.............	32–64	193	82	109	>250
Type II					
Susceptible........ ...	—	18 (1)	47 (3)	31 (3)	>200 (3)
Resistant........ .	8–16	39	44	30	84
Type III					
Susceptible...........	—	24 (1)	53 (3)	63 (3)	176 (3)
Resistant.............	16–32	42	64	117	171
Optochin					
Type I					
Susceptible...........	—	14 (2)	12 (3)	65 (3)	>250 (3)
Resistant.............	16–32	159	35	128	>250
Type II					
Susceptible...........	—	14 (2)	58 (2)	48 (3)	>180 (3)
Resistant.............	8–16	18	53	45	>180
Type III					
Susceptible...........	—	12 (2)	75 (2)	89 (3)	200 (3)
Resistant.............	16–32	16	67	84	158
Sulfonamide					
Type I					
Susceptible...........	—	23 (2)	13 (3)	63 (3)	>250 (3)
Resistant.............	8–16	25	35	84	>250
Type II (Schmidt)					
Susceptible...........	—	6 (2)	72 (3)	65 (3)	187 (3)
Resistant.............	8–16	7	125	74	195

* Figures in parentheses indicate mg of cells used in the particular experiment.

Acriflavine. The data for acriflavine-resistant organisms as given in table 2 show a significant difference in glucose and lactate dehydrogenase activity for type I, the most resistant of the three types; resistant type II organisms show delayed glucose activity only; and the resistant type III organisms show no difference in activity from the susceptible strains.

Atabrine. The glucose dehydrogenase activity of all three types of atabrine-resistant pneumococci is markedly reduced. This is the greatest difference found between susceptible and resistant strains. Whereas the susceptible strains can reduce methylene blue in the presence of glucose in 30 minutes or less, it requires $2\frac{1}{2}$ and more hours for reduction by the resistant strains. All three resistant strains also show a slight delay in lactate activity, but no change in glycerol activity.

Propamidine. As with the atabrine-resistant organisms, the glucose dehydrogenase activity of all the propamidine-resistant organisms is markedly weakened. The most resistant pneumococcus is type I, which requires more than 3 hours for the decolorization of methylene blue, whereas its susceptible parent strain can accomplish this reduction in 15 minutes. Glycerol activity of the resistant type I strain is also significantly reduced and types I and III likewise show reduced lactate dehydrogenase activity. The resistant type II organism manifests a greater endogenous activity than the susceptible parent strain. This may play a role by accelerating the dehydrogenation of a substrate. This possibility may therefore make the data difficult to interpret. At any rate, an accelerating effect of endogenous activity, if present, would obscure the inherent weakened activity of the resistant cells in all substrates. In other words, were it not for the accelerated endogenous activity of the resistant cells, the indicated reduction times in the presence of substrates would probably have been much greater than those of the susceptible cells.

Optochin. Among the optochin-resistant organisms only type I showed any significant decrease in dehydrogenase activity with glucose, glycerol, and lactate as hydrogen donators.

Sulfonamides. Data for the sulfonamide-resistant organisms include only types I and II (Schmidt) since these were the only available mucoid strains which were resistant to sulfonamides. In both cases we find a significant difference with respect to their glycerol dehydrogenase activity.

Table 3 represents an extension of the comparison with type I organisms using hexose diphosphate and ethanol as substrates. Except for the sulfonamide-resistant one, HDP activity is markedly decreased in all resistant forms. Ethanol activity is significantly decreased in all, but more so in the organisms resistant to optochin, acriflavine, and atabrine. This extension was made with type I organisms because these organisms manifested the highest degree of resistance and were most subject to decreased dehydrogenase activities.

These observations are presented in a comparative manner in table 4. An index of activity has been estimated for each of the resistant organisms. This index represents the ratio of t_r for the resistant organism to t_r for the susceptible. Thus, a ratio of 1 indicates the same reduction time for both susceptible and

resistant; a ratio of 2 would mean that the susceptible has twice the activity of the resistant, and so on. It can be seen that the atabrine-resistant organisms present the most consistent data for all types with marked loss of glucose dehydro-

TABLE 3

A comparison of dehydrogenase activities of susceptible and resistant mucoid pneumococcus, type I, in ethyl alcohol and hexose diphosphate

ORGANISM (2 MG)	METHYLENE BLUE REDUCTION TIME (MINUTES)		
	Ethyl alcohol	Hexose diphosphate	
	0.1 M	0.002 M	0.005 M
Parent (susceptible)..	7	24	11
Acriflavine-resistant	19	180	83
Atabrine-resistant	36	>250	183
Propamidine-resistant . ;	13	>250	39
Optochin-resistant	50	187	89
Sulfonamide-resistant	12	31	

TABLE 4

Methylene blue reduction time (t_r) indices for resistant mucoid pneumococci

SUBSTRATE	TYPE	t_r INDEX FOR ORGANISMS RESISTANT TO				
		Acriflavine	Atabrine	Propamidine	Optochin	Sulfonamide
Glucose (0.001 M)	I	3.57	16.43	12.87	11.36	1.09
	II	2.00	>17.00	2.17	1.28	1.16
	III	1.03	4.84	1.75	1.33	
Glycerol (0.1 M)	I	0.81	1.18	2.73	2.92	2.69
	II	1.09	1.29	0.94	0.92	1.75
	III	0.89	0.77	1.21	0.89	
Lactate (0.1 M)	I	2.07	1.46	1.53	1.97	1.33
	II	1.06	1.53	0.97	0.94	1.14
	III	1.01	1.84	1.87	0.94	
HDP (0.002 M)	I	7.50	>10.00	>10.00	7.79	1.29
(0.005 M)		7.55	16.55	3.55	8.09	
Ethanol (0.1 M)	I	2.71	5.14	1.86	7.16	1.72

$$t_r \text{ index} = \frac{t_r \text{ resistant organism}}{t_r \text{ susceptible organism}}.$$

genase activity, slight loss of lactate activity, and no loss of glycerol activity. Wherever there is a change in acriflavine- and propamidine-resistant organisms, there follows a suggested similarity in behavior to those resistant to atabrine. The distribution and degree of change with HDP as substrate follows the pattern obtained with glucose as substrate. The values for two concentrations of HDP

are given. With the exception of the propamidine-resistant organism the same degree of delay is found in both concentrations. The propamidine-resistant organism shows increased activity when the concentration of HDP is increased (table 4). It must also be noted that resistant type I organisms, in general, appear to be more susceptible to variation in dehydrogenase activities as compared to type III, of which the resistant form seems to show least variability in this respect.

TABLE 5

Comparison of dehydrogenase activity of pneumococci showing phase variation

ORGANISM	INCREASE (FOLD) IN RESISTANCE	METHYLENE BLUE REDUCTION TIME (MINUTES)								
		Glucose (0.001 M)		Glycerol (0.1 M)		Lactate (0.1 M)		Endogenous (no substrate)		
		mg of organisms used								
		1	2	2	3	2	3	1	2	3
Type II—Parent (susceptible), mucoid	—		10	59			48			>250
Type II—Drug-free, rough	32–64 (optochin) / 4–8 (sulfathiazole)		11		>250		>250			>250
Type II—Optochin-resistant, rough	32–64		19		>180		>180			>180
Type II—Sulfonamide-resistant, smooth	64		13	29			33			>250
Type III—Parent (susceptible), mucoid	—	31	11	93	57		74	∞	∞	>250
Type III—Drug-free, rough	16–32 (atabrine) / 2 (sulfathiazole)	25	9	38	6	78	5	126	62	5
Type III—Atabrine-resistant, rough	16–32	∞	138	105	2	>250	3	∞	102	8
Type III—Sulfonamide-resistant, smooth	32–64		35		∞		∞	∞	∞	∞

Dehydrogenase activity of phase variants. As previously mentioned, various resistant pneumococci were obtained which appeared to be rough variants. Dehydrogenase activities of the rough variants of parent drug-susceptible organisms were compared with a rough variant derived from type II which had been exposed to optochin and a rough type III exposed to atabrine. The degree of resistance to the respective drugs to which the latter organisms had been exposed appears to be of an independent nature, inasmuch as the rough forms that were never exposed to the drug shared the same degree of resistance. The activities of the two sulfonamide-resistant smooth strains, previously described, were also determined, but, unfortunately, equivalent smooth strains that had never been exposed to the drug were not available for comparison.

It can be seen from table 5 that the rough variant derived from the type II

mucoid strain without exposure to the drug has lost its lactate and glycerol activity but not its glucose activity. Since the rough organism derived from the mucoid strain as a result of exposure to optochin shows similar behavior, this loss of activity may not be attributed to an effect resulting from exposure to the drug. There is, however, a slight loss in the glucose dehydrogenase activity of the rough variant derived from drug exposure that is not shared by the rough equivalent obtained without exposure to drug. The corresponding drug-free rough variant of type III reveals the anomaly of extreme endogenous activity, which the rough organism derived by exposure to atabrine also possesses. The latter's activity, however, could be weakened by dilution of the bacterial suspension so that with 1 or 2 mg of organisms the activity shown in the presence of the substrate is the same or even less than that in the absence of substrate. The nature of the factors responsible for these anomalous activities are at present unknown to us. Previous studies on the respiratory activities of virulent and nonvirulent pneumococci have manifested similar variations (Sevag, 1933; Sevag and Maiweg, 1934).

When the type II sulfonamide-resistant smooth strain was compared with the susceptible mucoid parent strain, no loss in activity was observed; in fact, there was an acceleration in activity of the resistant organism in the presence of glycerol and lactate. The type III sulfonamide-resistant smooth strain shows a complete loss of activity in lactate and glycerol and a measurable decrease in activity in glucose. Since drug-free smooth forms of types II and III were not available for comparison with the respective resistant smooth forms, the analysis remains incomplete and the interpretation inconclusive.

DISCUSSION AND CONCLUSIONS

With exposure to various drugs, the pneumococci have developed a tolerance to the respective drugs along with an alteration, in varied instances, in their enzymatic activities. These resistant organisms show lowered dehydrogenase activity. This modification of activity is related to the types of pneumococci and the substrates tested; it does not represent a uniform pattern for all the strains that have developed resistance to various drugs. Resistant type I organisms, for example, exhibit greater tendency toward loss of dehydrogenase activity, whereas the resistant type III organisms are less inclined to deviate from the normal pattern of the parent. These differences are discussed in the text (see also tables 2 and 4). Since the normal activities of the three types of pneumococci investigated are in themselves variable, this varied tendency toward loss of activity upon exposure to drugs may, in effect, be a reflection of varied metabolic capacities of the different organisms. These findings emphasize the hazards that may be involved in studies which attempt to draw generalizations from experiments performed with single strains of organisms.

In general, there appears to be a similarity in response between the two acridines and propamidine. Also, it was with these that significant cross resistance was demonstrated.

The sulfonamide-resistant organisms (mucoid) show no change in glucose and lactate activity but measurable delay in glycerol activity. MacLeod (1939),

studying sulfapyridine-resistant pneumococci (type I), also found no change in glucose activity but loss of glycerol activity in comparison with the susceptible parent organism. However, he also reported loss of ability to reduce methylene blue in the presence of pyruvate and lactate as substrates. Under the conditions of our experiment, pyruvate could not act as substrate for normal organisms and no change in the lactate activity of the resistant organisms could be found. We are unable to give any explanation for these discrepancies.

Two of the three optochin-resistant organisms retained normal activity. Only the resistant type I organism was affected, and this in all substrates (table 4). Because of the variability of this response, interpretation must remain inconclusive. This is only another manifestation of the greater variability of the resistant type I organisms to the loss of dehydrogenase activity.

Since HDP and ethyl alcohol were used as substrates only with the type I organisms, no generalizations with respect to the other types can be made with these substrates. However, it may be significant that loss in HDP activity followed the same pattern as loss in glucose activity for the type I organisms. All but the sulfonamide-resistant organism exhibited a marked decrease in activity.

The data presented here may be of some help to characterize the nature of resistance and the mode of action of the drug. An interpretation of the mechanisms of the observed resistance phenomena can at present be only in the nature of suggestions based on the analysis of the data presented here and of previously reported investigations (Sevag, 1946). Susceptibility to drugs must depend upon an interference with or a disruption of chemical reactions involved in the physiology of the organisms. Unhindered functioning of the physiological activities of the resistant organisms must depend on metabolically active sites not susceptible to the action of drugs.

Since it is known that the oxidative enzymes of bacteria are subject to inhibition by these drugs, the development of resistance may involve either a reduction in the degree of the activity of drug-sensitive enzymes or a certain alteration in the properties of these enzymes, resulting in their loss of affinity for a drug and a related loss of oxidative activity as well. The resistant organism would need to utilize alternate metabolic processes that are not susceptible to the action of the drug for the maintenance of their modified physiological functions (Sevag, 1946; Sevag and Green, 1944). Considering the flavoprotein system as an example and, as previously stated, the most likely one involved, a depletion or alteration of either the protein or coenzyme group would lead to lowered activity and to the elimination of susceptibility to the action of drugs. An attempt toward the resolution of this problem will be presented in succeeding studies.

SUMMARY

The development of resistance of pneumococci (types I, II, and II) to the inhibitory action of acriflavine, atabrine, propamidine, optochin, and sulfonamide is described. Data are given as to (a) the degree of resistance obtained; (b) the

number of transfers in the drug required for the development of the maximum degree of resistance; (c) the stability of resistance once obtained; (d) the production of smooth and rough phase variants; (e) cross resistance; and (f) a comparison of dehydrogenase activities.

A marked degree of cross resistance occurred between propamidine- and acriflavine-resistant organisms in that organisms made resistant to propamidine were also resistant to acriflavine and vice versa. The atabrine-resistant organisms shared a lesser degree of cross resistance with the acriflavine- and propamidine-resistant pneumococci. Pneumococci resistant to the other drugs were only resistant to the drug to which they were exposed.

By conventional Thunberg techniques, dehydrogenase activities of the parent susceptible and drug-resistant pneumococci were determined in the presence of glucose, glycerol, lactate, hexose diphosphate, and ethyl alcohol. Pyruvate, succinate, maleate, and fumarate were inactive as substrates. All atabrine-resistant mucoid organisms showed a marked decrease in glucose activity, significant loss of lactate activity, and unaltered glycerol activity. Sulfonamide-resistant mucoid pneumococci were altered only by a reduced glycerol activity. Propamidine-resistant pneumococci all showed decreased glucose activity; however, only resistant type II organisms were unaltered in lactate activity; and only resistant type I organisms showed decreased glycerol activity. Acriflavine-resistant pneumococci were more varied in type I, showing decreased glucose and lactate dehydrogenase activities; type II showed only decreased glucose activity; and type III was unaffected. Only the type I optochin-resistant organisms exhibited decreased activity and this in all substrates tested. Hexose diphosphate and ethyl alcohol activity of the resistant type I organisms (except sulfonamide) was significantly decreased in varying degrees.

The activities of a few phase variants were examined but the results presented anomalies which could not be satisfactorily analyzed.

The mechanism of the altered dehydrogenase activities of the resistant pneumococci and its relationship to the mechanism of the resistance to drugs are discussed.

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ENZYMATIC STUDIES ON THE MECHANISM OF THE RESISTANCE OF PNEUMOCOCCUS TO DRUGS

II. The Inhibition of Dehydrogenase Activities by Drugs; Antagonistic Effects of Riboflavin to Inhibitions[1]

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In the preceding study (Gots and Sevag, 1948) it was shown that pneumococci made resistant to various drugs frequently possessed diminished dehydrogenase activities. It was suggested that the decreased dehydrogenase activity of the particular resistant organisms was a manifestation of a deficiency of susceptible sites, i.e., the resistant organisms depended on nonsusceptible metabolic sites for the maintenance of their physiological activities. The effect of the drugs on the dehydrogenase activity in the presence of the various substrates was therefore investigated to obtain information concerning these questions and to characterize certain of the enzymes involved in the production of the observed phenomena.

Inhibition of Dehydrogenase Activity

Methods and results. For the determination of the methylene-blue-reducing ability of the organisms a refinement of the technique used by Schnabel (1920) was used. This was substituted for the conventional Thunberg method, previously used, in order to facilitate the performance of simultaneous experiments with several substrates and varying concentrations of drugs, organisms, and other additions. Since some experiments involved 100 or more combinations, the use of Thunberg tubes would have been prohibitive. The technique, in effect, is a microadaption with some experiments being conducted with as little as 0.05 mg of organisms. The results obtained by this method are qualitatively, but not quantitatively, comparable to those obtained by the Thunberg technique.

The bacterial suspension and the reagents used were prepared as previously described (Gots and Sevag, 1948). Using small serological tubes (10-by-100-mm), the system consisted of 0.05 ml of methylene blue (0.001 M in M/20 phosphate buffer at pH 7.6), 0.1 ml of substrate, 0.1 ml of drug, bacterial suspension (0.1 mg per 0.1 ml), and M/20 phosphate buffer (pH 7.6) to give a total of 1 ml. The bacterial suspension was added last, the contents were immediately mixed, and 1 ml of melted petrolatum was carefully layered over the mixture. The tubes were then placed in a water bath at 37 C and the time in minutes required for 100 per cent reduction of the methylene blue was recorded.

Inhibition of dehydrogenase activities of drug-susceptible parent organisms. Since all of the susceptible types were affected by the drugs in a similar manner, data are presented (table 1) for a single type. The results from typical experiments are given in table 1 for glucose, hexose diphosphate (HDP), and ethyl

TABLE 1

The effect on dehydrogenase activity of pneumococcus, type I, of acriflavine, atabrine, propamidine, optochin, and sulfonamide in the presence of glucose, hexose diphosphate (HDP), ethyl alcohol, glycerol, and lactate

DRUG	DRUG CONCENTRATION (×10⁻⁴)	GLUCOSE (0.001 M)		HDP (0.003 M)		ETHYL ALOCHOL (0.1 M)		GLYCEROL (0.1 M)	LACTATE (0.1 M)
		Mg of organisms used							
		0.2	0.1	0.3	0.2	0.2	0.1	0.5	0.5
		Reduction time in minutes							
Acriflavine	1.0	17	85	21	95	∞	∞	39	54
	0.5	14	50	21	95	∞	∞	30	48
	0.25	11	27	21	95	>120	>120	30	40
	0.00	8	18	11	19	22	38	29	38
Atabrine	4.0			27	95	∞	∞		
	2.0	24	∞	21	68	∞	∞	43	68
	1.0	18	∞	21	32	120	150	35	66
	0.5	13	26					27	40
	0.00	8	18	11	19	22	38	33	45
Propamidine	10.0	14	∞	10	16	31	38	>120	75
	5.0	11	28	10	17	43	38	82	35
	2.5	10	21	10	16	37	38	66	32
	0.00	9	19	11	19	22	38	40	34
Optochin	10.0	∞	∞	∞	∞	∞	∞		
	5.0	∞	∞	22	72	∞	∞	45	37
	2.5	27	∞	14	28	51	150	30	35
	1.25	17	85	11	20	35		28	32
	0.625	14	34					28	29
	0.00	9	20	11	19	22	38	28	29
Sulfathiazole (Na salt)	40.0	8	19	8	16	22	35	28	48
Sulfapyridine	4.0	8	22	12	21	25	37	30	48
	0.00	8	20	11	19	22	38	30	48

alcohol with two different weights of organisms. The conditions chosen for HDP and ethyl alcohol were those under which normal activities approached that of glucose. Glycerol and sodium lactate dehydrogenase activities were consistently low, and the results shown are those obtained with 0.5 mg of organisms. A weight of organisms greater than this was impractical because of increased endogenous activity.

In all cases, the degree of inhibition of the methylene-blue-reducing ability by a given concentration of a drug was related to the weight of organisms used. Inhibition by the drugs occurred less readily in the presence of greater amounts of bacterial cells.

Glucose dehydrogenase was inhibited by all the drugs except the sulfonamides. Concentrations as high as 1:250 of sodium sulfathiazole and 1:2,500 of sulfapyridine did not inhibit the activity of any of the substrates. (Factors responsible for this effect are discussed below.) The two acridines, acriflavine and atabrine, inhibited the glucose dehydrogenase in comparatively high dilutions. Optochin required slightly higher concentrations, and propamidine even more, for the inhibition of glucose dehydrogenase activity. The activity in the presence of HDP is less inhibited than that in the presence of glucose, and it is not at all affected by propamidine. Ethyl alcohol activity is also little affected by propamidine, but acriflavine exerts its greatest inhibition in the presence of this substrate. Glycerol and lactate activity are only slightly affected by the drugs, with propamidine being the most inhibitory. Concentrations higher than those shown for propamidine, optochin, and sulfapyridine could not be obtained because of insolubility in the phosphate medium. Higher acriflavine concentrations resulted in the clumping of the organisms.

The amount of drug required to inhibit dehydrogenase activity is much greater than that required to inhibit growth, as given in the preceding paper. However, the number of organisms required (300 to 400 million) to give demonstrable dehydrogenase activity is far greater than the number of organisms (80 to 90 million) present in the typical inocula of the growth systems. Since the dehydrogenase activity and its degree of inhibition by the drugs are dependent upon the number of organisms present, this would explain the difference in the amount of agent required for the inhibition in the two systems. In experiments conducted with 0.05 mg of organisms (150 million cocci) or in syst ems with a particularly low activity, glucose dehydrogenase activity could be inhibited by atabrine concentrations as low as 1:160,000. This is of the same order of magnitude as the growth-inhibiting concentration of atabrine of 1:400,000.

With glucose, the substrate concentration is also a factor determining the degree of inhibition obtained with at least atabrine, propamidine, and, to a lesser extent, acriflavine. In an experiment with the type III organism, the following results were obtained in 0.1 M and 0.001 M of glucose with 0.1 mg (300 to 400 million) of bacterial cells (table 2). Thus, the inhibition is smaller in higher concentrations of glucose. This could only be examined with glucose as substrate since the activities in the presence of the other substrates are dependent on an optimal substrate concentration, and the degree of inhibition is dependent on the activity manifested under optimal conditions.

The failure of sulfonamides to inhibit the activity in the presence of any of the substrates was confirmed by use of the Thunberg technique. Concentrations as high as 1:160 of sodium sulfathiazole were completely ineffective. As will be noted in the discussion, this lack of inhibition is due to the antagonism of methylene blue.

The ability of propamidine to inhibit glucose activity but not HDP activity might indicate an interference with phosphorylation mechanisms. This would be more decisive if the exact nature of the two dehydrogenase systems were better defined. It is yet to be determined whether glucose must first be phosphorylated in order to serve as a hydrogen donator in these dehydrogenase systems or whether it can be directly oxidized to, e.g., gluconic acid without phosphorylation. In Thunberg experiments, however, some inhibition of HDP activity by propamidine could be obtained with 1:1,600 concentration. Inhibition by propamidine was found to be peculiarly dependent on the order of addition of various agents. When the drug was added with the substrate from the side arm of Thunberg tubes after 10 minutes' incubation, the inhibition of glucose activity was less than when the drug was allowed to incubate in the

TABLE 2

The effect of glucose concentration on the inhibition of glucose dehydrogenase of pneumococcus, type III, by acriflavine, atabrine, and propamidine

	PROPAMIDINE ($\times 10^{-4}$)			ACRIFLAVINE ($\times 10^{-4}$)			ATABRINE ($\times 10^{-4}$)			CONTROL
	10	5	2.5	1.0	0.5	0.25	0.5	0.25	0.125	0.00
	Methylene blue reduction time (minutes)									
Glucose 0.1 M	∞	43	20	∞	50	45	∞	50	33	16
Glucose 0.001 M.. .	∞	∞	68	∞	90	45	∞	∞	∞	16

presence of the organisms before substrate was added alone from the side arm. Since in the experiments using the Schnabel technique the organisms are added last, this may well explain the failure of HDP inhibition by propamidine when determined by this method.[3]

At any rate, the inhibition of glucose activity in any system is greater than that of HDP activity by propamidine, as well as by atabrine. The possible action of these drugs on the phosphorylation mechanisms will require further study. Atabrine has already been implicated in the interference with these systems (Speck and Evans, 1945; Bovarnick, Lindsay, and Hellerman, 1946; Marshall, 1948a).

Effects of the drugs on the dehydrogenase activity of resistant organisms. Inhibition of the dehydrogenase activities of the resistant organisms was studied in order to determine if the resistance to inhibition of growth was associated with a resistance to inhibition of dehydrogenase activity by the drugs.

Using the Schnabel technique, a comparison of the inhibitions of glucose

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[3] Propamidine possesses another property that might add to its dehydrogenase-inhibiting action. In Thunberg experiments, if high concentrations of propamidine are added from the side arm after the organisms have reduced the methylene blue in the presence of glucose, the leuco form is slowly reoxidized to the blue form. This would indicate a competition between methylene blue and propamidine for the hydrogen mobilized from the substrate, which would explain the observed decrease in inhibition in the presence of increased substrate.

dehydrogenase activity for susceptible and resistant organisms was made. Only the glucose dehydrogenase activity was studied in these experiments because this was the most readily inhibited and, as previously reported (Gots and Sevag, 1948), the most frequently decreased in resistant organisms. This difference in the glucose activity of the susceptible and resistant organisms in the absence of the drugs presented difficulties when the degrees of inhibition of the activities of

TABLE 3

Inhibition of glucose dehydrogenase of pneumococci by acriflavine, atabrine, propamidine, and optochin

(A comparison of reduction time in minutes for susceptible and resistant organisms in glucose, 0.001 M)

DRUG	DRUG CONCENTRATION (×10⁻⁴)	TYPE I				TYPE II				TYPE III			
		Susceptible		Resistant		Susceptible		Resistant		Susceptible		Resistant	
		Mg of organisms used											
		0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1
		reduction time in minutes											
Acriflavine	1.0	17	85	44	∞	∞	∞	21	115	25	∞	67	∞
	0.5	14	50	18	∞	26	∞	18	52	18	45	28	∞
	0.25	11	27	14	76	22	65	16	43	15	28	18	70
	0.00	8	18	11	27	10	29	12	38	8	16	16	44
Atabrine	2.0	24	∞	94	∞	∞	∞	∞	∞	∞	∞	∞	
	1.0	18	∞	77	∞	∞	∞	73	∞	27	∞	40	∞
	0.5	13	26	60	∞	35	∞	24	∞	21	∞	30	∞
	0.25					20	∞	21	∞	18	49	18	∞
	0.00	8	18	38	∞	9	28	20	52	8	16	16	43
Propamidine	10.0	12	∞	17	∞					17	∞	12	30
	5.0	10	40	16	47					9	29	12	29
	2.5	9	21	16	33					8	17	12	27
	0.00	9	20	16	33					7	16	12	27
Optochin	5.0	∞	∞	∞	∞								
	2.5	27	∞	50	∞								
	1.25	17	43	28	∞								
	0.625	14	34	26	78								
	0.00	8	24	22	50								

the two organisms were compared. Since the degree of inhibition is related in an inverse manner to the ability of the bacterial suspensions to reduce methylene blue, a decreased activity will naturally show greater susceptibility to inhibition. Therefore, in comparing the effect of the drugs on dehydrogenase activities, a similar base line of control activity had to be made in most instances. This could be accomplished by increasing the number of the resistant organisms in the comparative test systems so that their control activities approached the activities characteristically demonstrated by fewer susceptible organisms.

Table 3 presents data from typical experiments permitting this adjustment.

For example, in type III resistant organisms the control activity of 0.2 mg cells is approximately that of 0.1 mg of the susceptible organisms, and the inhibition of activity by the respective drugs is much greater for the susceptible than for the resistant organisms. The effect of an increase in the number of organisms, irrespective of activity, on the degree of inhibition obtained may well be a significant factor. However, in several cases when, in control systems, the resistant cells do not show a greatly decreased activity, a comparison of activities obtained with the same number of susceptible and resistant cells can be made. For example, the inhibition of type II cells by acriflavine shows that, whether one compares the activities of (a) 0.2 mg of susceptible cells with those of 0.2 mg resistant cells, or (b) 0.1 mg of susceptible cells with that of 0.2 mg resistant cells, or (c) 0.1 mg of susceptible cells with that of 0.1 mg of resistant cells, the dehydrogenase activities of the resistant cells are markedly more resistant than those of the susceptible cells. The results with type II atabrine cells show that 0.2 mg of resistant cells, with an activity slightly greater than that of 0.1 mg susceptible cells, are more resistant. Two-tenths mg of type I atabrine-resistant organisms manifest a greatly decreased methylene blue reduction time of 38 minutes. In contrast, 0.1 mg of the respective susceptible cells required 18 minutes for the same performance. Despite this difference in activity, the resistant organisms were inhibited only 60 per cent by a 1:5,000 concentration of atabrine, whereas the susceptible organisms were completely inhibited by a 1:10,000 concentration of the drug. This relationship is indicated also by types I and III propamidine-resistant cells. For example, 0.2 mg of propamidine-resistant type I cells having a control activity comparable to that of 0.1 mg of susceptible cells show strong resistance to propamidine. This relationship can similarly be seen with type III propamidine-resistant and type I optochin-resistant cells.

With the possible exception of the type I acriflavine-resistant organism, all of the resistant pneumococci are thus less subjected to inhibition of their glucose dehydrogenase activity by the respective drugs than are the corresponding susceptible parent stains.

Acridine and diamidine cross resistance to inhibition of dehydrogenase activity. In the preceding study, the rationale for the inclusion of the aromatic diamidine, propamidine, was based upon published observations of a similarity of action between this compound and the acridines. This similarity of action was extended in the observations (a) that a cross resistance to inhibition of growth between the two compounds existed; and (b) that loss of dehydrogenase activity by the pneumococci made resistant to these agents followed a similar pattern. Since the resistant organisms are also resistant to glucose dehydrogenase inhibition, a study was made of the cross resistance in this respect.

Using the same methods, the inhibition of glucose dehydrogenase activity by all three drugs was determined for the parent cells which were resistant and susceptible to acriflavine, atabrine, and propamidine. This comparison was made with all types with similar results, but, because of the large variations of control activities, a comprehensive comparison was only possible with the type III organisms. With these organisms a common range of activity could be obtained with a twofold increase in the number of the resistant organisms.

Table 4 presents the results compared on a common basis of a drug-free activity of 12 to 16 minutes methylene blue reduction time. The results are given in percentages of inhibitions calculated from reduction times (e.g., with 12 minutes as the reduction time for the control, 24 minutes in the presence of drug would correspond to a 50 per cent inhibition). With the exception of the atabrine-resistant organisms in propamidine, all the resistant organisms are less inhibited than the parent susceptible organisms. Organisms made resistant to one drug exhibit a resistance to the inhibition of dehydrogenase activity not only by that drug but also by the drugs to which they never had been exposed. This cross resistance is manifested more between acriflavine and propamidine than between atabrine and the others. A similar relationship existed between the cross resistance to the inhibition of growth.

TABLE 4

Inhibition of glucose dehydrogenases by atabrine, acriflavine, and propamidine of susceptible type III and corresponding resistant pneumococci

DRUG	DRUG CONCEN-TRATION (×10⁻⁴)	SUSCEPTIBLE (0.1 MG CELLS)	ACRIFLAVINE-RESISTANT (0.2 MG CELLS)	ATABRINE-RESISTANT (0.2 MG CELLS)	PROPAMIDINE-RESISTANT (0.2 MG CELLS)
		Percentage of inhibition			
Acriflavine	1.0	100	76	80	38
	0.5	65	28	34	16
	0.25	53	11	21	12
Atabrine	2.0	100	100	100	47
	1.0	88	45	60	33
	0.5	83	30	46	22
Propamidine	10.0	100	43	100	27
	5.0	54	28	38	8
	2.5	30	20	32	0

The activity of organisms in the absence of a drug was 12 to 16 minutes.

The Effects of Riboflavin

In order to determine the role of flavoprotein in the resistance phenomenon, experiments were performed with riboflavin to study its effects on the inhibition of growth and dehydrogenase activity by the drugs and on the impaired activity of the resistant strains.

Methods. The inhibitory effect of the drugs on growth systems was determined in the partially defined medium of Adams and Roe (1945) containing vitamin-free casein hydrolyzate (SMACO) instead of the "casamino acids." Various batches of media were prepared containing varying concentrations of riboflavin (Merck) added from stock solution in 0.02 N solution of acetic acid. The inhibition of growth was determined as previously described (Gots and Sevag, 1948).

Dehydrogenase experiments were performed by the Schnabel method. The vitamins, prepared freshly in phosphate buffer, were substituted for part of the

phosphate buffer in the final system. All other reagents and preparations remained as previously described.

Effect of riboflavin on the inhibition of growth. The minimal amounts of drug required to inhibit the organisms in the presence and absence of added riboflavin are given in table 5. The presence of riboflavin had no effect on the inhibition of growth by sulfathiazole and only slight effect on inhibition by optochin and propamidine. The inhibition by acriflavine and atabrine is affected more by the presence of riboflavin, in that higher concentrations of these drugs are required for inhibition of growth as the concentration of riboflavin is increased. For example, twice as much atabrine is required to inhibit type III organisms in the presence of 10 μg of riboflavin per ml, 4 times as much with 100 μg, and 8 times as much with 250 μg. The data given are based on 24-hour readings. The relief of inhibition by riboflavin was more pronounced with prolonged readings. By turbidimetric readings with the Klett photoelectric colorimeter, it was found that the relieving of inhibition by riboflavin could not have been due to a stimulatory action of the vitamin since its presence, per se, in the absence of drugs, had no such effect.

Effect of riboflavin on the inhibition of glucose dehydrogenase activity by the drugs. The inhibition of glucose activity by propamidine, acriflavine, and atabrine is also relieved by the presence of riboflavin. Table 6 gives the results for a typical experiment with 0.1 mg of susceptible type II organisms in 0.001 M glucose. The other types behaved similarly. The activity in the absence of the drug was stimulated as riboflavin concentration was increased. However, the effect of the added riboflavin on relieving the inhibition by propamidine and the acridines was quite disproportionate to what might have been expected on a stimulatory basis alone. The effect of the vitamin on inhibition produced by optochin is, however, of the same order as the stimulation which it shows in the absence of the drug. In this case, there does not appear to be any true antagonism. Nicotinamide and thiamine in concentrations equimolar to that of riboflavin were also tested for antagonistic effect in these systems and were found to be completely ineffective.

DISCUSSION AND CONCLUSIONS

Pneumococci possess dehydrogenase systems, particularly active on glucose as substrate, which are susceptible to the action of the drugs; the dehydrogenase activities of the resistant organisms, though reduced, are more resistant to the action of the drugs. This suggests that the susceptibility and resistance to inhibition of growth may well be a function of these systems. In the absence of well-defined information concerning the intermediary steps involved in normal dehydrogenase activity, as well as incomplete extension to other systems, peremptory conclusions cannot be drawn. However, since riboflavin is at least involved as an antagonist to the inhibition of both growth and dehydrogenase activity by the acridines and propamidine, the flavoprotein is offered as a site of action of these compounds.

A correlation of the pattern of decreased dehydrogenase activity of the resistant

TABLE 5

Antagonism by riboflavin to the inhibition of growth* of pneumococci by drugs

TYPE	DRUG	RIBOFLAVIN (µg/ML)			
		None	10	100	250
		Minimal inhibitory concentration of drug (µg/ml)			
I	Propamidine	6.25	12.50	12.50	12.50
	Acriflavine	1.25	2.50	2.50	5.00
	Atabrine	2.50	5.00	5.00	10.00
	Optochin	0.62	0.62	0.62	1.25
	Sulfathiazole	31.20	31.20	31.20	31.20
II	Propamidine	12.50	12.50	—	25.00
	Acriflavine	0.62	0.62	1.25	2.50
	Atabrine	5.00	5.00	—	40.00
	Optochin	2.50	2.50	2.50	2.50
	Sulfathiazole	15.60	15.60	15.60	7.80
III	Propamidine	12.50	12.50	12.50	25.00
	Acriflavine	2.50	2.50	5.00	10.00
	Atabrine	2.50	5.00	10.00	20.00
	Optochin	5.00	5.00	10.00	10.00
	Sulfathiazole	31.20	31.20	31.20	31.20

* Twenty-four-hour growth at 37 C.

TABLE 6

Effect of riboflavin on the inhibition of glucose dehydrogenase

(Type II susceptible pneumococcus, 0.1 mg.; glucose concentration = 0.001 M)

DRUG	DRUG CONCEN-TRATION	RIBOFLAVIN (µg/ML)			
		None	100	200	400
	µg/ml	reduction time in minutes			
Control	—	16	13	11	10
Propamidine	1000	∞	∞	∞	42
	500	∞	94	39	25
	250	24	20	18	12
Acriflavine	100	∞	∞	56	32
	50	44	31	23	16
	25	16	14	13	12
Atabrine	400	∞	∞	∞	∞
	200	∞	∞	∞	106
	100	90	50	32	24
Optochin	250	∞	∞	∞	∞
	125	45	43	38	28
	62.5	36	35	30	18
No glucose	—	∞	∞	∞	∞

Parallel tests with equimolar concentrations of nicotinamide and thiamine showed no effect.

organisms (Gots and Sevag, 1948) with the inhibition spectrum obtained in the presence of various drugs and substrates is desirable. Atabrine-resistant organisms showed a marked decrease in glucose, HDP, and ethyl alcohol dehydrogenase activity; slight decrease in lactate; and no change in glycerol. The inhibition of the corresponding dehydrogenase systems of the susceptible cells by atabrine corresponds to this pattern remarkably in that atabrine inhibits the dehydrogenation of glucose, HDP, and ethyl alcohol, but practically no effect on lactate and glycerol dehydrogenases was observed. Acriflavine and propamidine, in general, also follow this correlation of impaired dehydrogenase activity of resistant organisms and the susceptibility of the susceptible cells to the inactivation of dehydrogenase activities by these drugs. The results with propamidine in the inhibition experiments with HDP and ethyl alcohol present certain peculiarities which have been fully discussed in the text. The activities of all three of the susceptible organisms were similarly inhibited by optochin yet only the resistant type I organism showed any loss of activity. The present experimental data offer no explanation for this discrepancy.

MacLeod (1939) reported sulfapyridine inhibition of glycerol, lactate, and pyruvate dehydrogenase activities with type I pneumococcus. The dehydrogenase activities in all substrates examined by us were completely unaffected by high concentrations of the sulfonamides. Even though the glycerol activity of the parent strain was not inhibited by sulfonamides, the sulfonamide-resistant organisms did show a decrease in glycerol activity. The observations of Clifton and Loewinger (1943) may throw some light on this inconsistency. They found that the oxygen consumption and anaerobic glycolysis of *Escherichia coli* are inhibited by sulfonamides. However, the dehydrogenase activity measured by methylene blue reduction was not interfered with even with saturated solutions of sulfonamide. This observation was interpreted by Sevag (1946) to indicate that methylene blue competes effectively with sulfonamide for flavoprotein and therefore no inhibition of methylene blue reduction could be observed. These observations can be analogously applied to the pneumococci in that respiration of type I pneumococci is inhibited by sulfathiazole up to 40 per cent (Sevag and Shelburne, 1942; Sevag, Richardson, and Henry, 1945) and, as is found in the present study, the dehydrogenase activity in the presence of methylene blue is not affected in the highest concentrations of sulfathiazole. Eyster (1942) found that sulfanilamide in comparison to other narcotics is extremely potent in its effect on the adsorption of methylene blue by charcoal particles.

In a preliminary study, the antagonistic effect of methylene blue on the inhibition of aerobic respiration by sulfonamide was determined in the Barcroft-Warburg apparatus. The system contained 1 mg of pneumococcus, type I, containing catalase, 0.03 M glucose, and M/20 phosphate buffer of pH 7.6. The following results were obtained: the control had a QO_2 value of 250; with 3.8×10^{-3} M sulfathiazole there was 61 per cent inhibition. This inhibition with 1.67×10^{-4} M methylene blue was reduced to 6 per cent. Methylene blue alone also showed 54 per cent inhibition.

Although the interrelationship of the findings above is being further investi-

gated, the results of similar experiments with the Thunberg technique on sulfonamide, methylene blue antagonism can be reported as conclusive. In three systems in which the order of the addition of various factors was varied, only the system in which methylene blue was added last from the side arm of the Thunberg tube showed 57 per cent inhibition of the reduction of the dye by sulfathiazole. This inhibition indicates that sulfonamides and methylene blue compete for the same enzyme site.

On the basis of the antagonistic effect of riboflavin on the inhibition of both growth and dehydrogenase activity, and the failure to produce these effects by thiamine and nicotinamide, there would seem to be a definite correlation between flavoprotein metabolism and resistance to acriflavine, atabrine, and propamidine. The action of optochin cannot be so correlated from the present findings, and, in view of methylene blue antagonism, the data with sulfonamides are as yet inadequate and require further detailed study.

The competition of the acridines with riboflavin can be postulated on the basis of structural similarity. Propamidine has an action similar to that of the acridines, in many respects, yet bears no structural similarity to either the acridines or riboflavin. Conversely, the two acridines show points of dissimilarity in behavior in spite of similar structure. It would appear, therefore, that structural similarity is not an absolutely essential factor for the accomplishment of the interferences observed. Yet, irrespective of the nature of the competition, the fact that a dimensional similarity between an inhibitor and competing metabolite does exist would naturally result in an enhancement of the interference. The interference by agents which are dissimilar in structure would depend on other properties which could still be associated with the competition. The structural analogues, in addition to the property of similar configuration, may also share these other properties. In the present study, the acridines and propamidines show a common property of basicity. The cationic nature of these compounds has led to an explanation of their mode of action on the basis of competition with hydrogen ions for vital enzyme positions (Browning, Gulbransen, and Kennaway, 1919; Elson, 1945; Albert et al., 1945; Massart et al., 1947b). On the basis of the observed inhibitions of respiration by acridines (Silverman, Ceithaml, Taliaferro, and Evans, 1944; Ferguson and Thorne, 1946; Massart et al., 1947a; Deley, Peeters, and Massart, 1947; Marshall, 1948a) and by diamidines (Bernheim, 1943; Marshall, 1948b; Wien, Harrison, and Freeman, 1948), the negatively charged enzyme protein for which the drug cation and hydrogen ions compete is probably the dissociating acidic group of a respiratory enzyme. Quastel and Wheatley (1931) have suggested that the dehydrogenase system is primarily of an acidic nature on the basis that basic dyes inhibited and the acidic dyes did not inhibit the dehydrogenase activity. This would be in agreement with the observations which have been presented. However, the competition of an ionizable enzyme hydrogen with a cation of a drug, as discussed previously (Sevag, 1946), does not explain the lack of such a competition in a resistant organism.

SUMMARY

The effects of acriflavine, atabrine, propamidine, optochin, and sulfonamides on the dehydrogenase activities of pneumococci (types I, II, and III) in the presence of glucose, hexose diphosphate (HDP), glycerol, lactate, and ethyl alcohol were investigated.   The glucose dehydrogenase activities of the parent susceptible pneumococci were inhibited by all the drugs except sulfonamides. The latter had no effect on dehydrogenase activity in the presence of any of the substrates investigated.   This effect has been found to be due to a competition between sulfathiazole and methylene blue.   Dehydrogenase activity is less inhibited in the presence of HDP than in the presence of glucose by acridines and optochin, and it is not at all affected by propamidine.   Ethyl alcohol activity is also little affected by propamidine but is inhibited by the acridines and optochin. Glycerol and lactate activity are only slightly affected.

The degrees of inhibition by drugs are related to the control activities of the organisms, the number of organisms present in the systems, and, in the case of glucose, the substrate concentration.

The drug-resistant pneumococci are less subject to the inhibition of their glucose dehydrogenase activity by the respective drugs than are the corresponding susceptible parent strains.   A cross resistance to inhibition of glucose dehydrogenase exists between organisms made resistant to the acridines and to propamidine.

Riboflavin can counteract the inhibitory effect of acriflavine and atabrine on the growth of the susceptible organisms.   It is only slightly antagonistic to propamidine and optochin in the same systems and not at all to sulfonamides.

The inhibition of glucose dehydrogenase activity by the acridines and propamidine is also relieved by the presence of riboflavin, but not by nicotinamide or thiamine.   The inhibition of the glucose dehydrogenase by optochin is unaffected by any of the vitamins.

The findings are analyzed and discussed in relation to (a) similarity of the physical properties and action of structurally dissimilar acridines and propamidine; (b) the effects of these drugs on dehydrogenases in relation to their mode of antibacterial action; and (c) the antagonism of riboflavin to inhibition by the drugs.

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ENZYMATIC STUDIES ON THE MECHANISM OF THE RESISTANCE OF PNEUMOCOCCUS TO DRUGS

III. Experimental Results Indicating Alteration in Enzyme Proteins Associated with the Development of Resistance to Drugs[1]

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In the preceding articles (Gots and Sevag, 1948; Sevag and Gots, 1948) it was demonstrated that pneumococci made resistant to various drugs manifest decreased dehydrogenase activities toward glucose, hexose diphosphate, glycerol lactate, and ethyl alcohol as hydrogen donators. This effect is not shared uniformly and to the same degree by all three types of pneumococci, each made resistant to various drugs. In the preceding studies, no attempt was made to determine the extent of the reduction of the dehydrogenase activities of resistant pneumococci when hydrogen donators other than those mentioned above were used. Under these conditions, the degree of resistance manifested by pneumococci during growth in the presence of various drugs cannot, at present, be accurately related to the decreased dehydrogenase activities.

The results presented here, however, are clearly indicative of certain basic changes in properties which are manifested by drug-resistant pneumococci in response to the action of drugs and which are responsible for the decreased dehydrogenase activities. The particular strain which was used as the test object for further information was a type I pneumococcus. Type I appears to be capable of experiencing the most widely distributed impairment in dehydrogenase activity. For example, type I organisms resistant to atabrine and acriflavine show decreased dehydrogenase activity on glucose and lactate; propamidine- and optochin-resistant organisms are less active in glucose, lactate, and glycerol; and sulfonamide-resistant ones in glycerol only. With the exception of the sulfonamide-resistant organisms, all of the resistant type I organisms also showed decreased activity in hexose diphosphate and ethyl alcohol. Also, since, of the various drugs studied, atabrine was capable of crippling glucose dehydrogenases of all three types of pneumococci to the greatest degree, a strain of atabrine-resistant type I pneumococcus was utilized for this study.

The experiments performed deal with the following points: (a) the question of cell permeability with respect to the decreased dehydrogenase activities of drug-resistant cells; a study of the activities of cell-free enzyme systems, their susceptibility to a drug, and their response to excess riboflavin; (b) the effect of

diminishing the number of cells on the activity of a system, and the response of these cells to excess riboflavin; (c) the effect of temperature on the activity of cells and their response to excess riboflavin; and (d) the riboflavin contents of cells in relation to their normal and decreased dehydrogenase activities.

EXPERIMENTAL RESULTS

The techniques used in these experiments have been described in the preceding articles and need not be repeated here.

Study of cell-free extracts of susceptible and resistant cells. The question of whether or not the resistance to a drug could be due to an altered cell wall (in the resistant organisms) was studied. If the permeability of the cell wall undergoes alteration during the development of resistance, decreased permeability to the drug and the substrates could explain the increased resistance and decreased dehydrogenase activities as well. To obtain an answer to this question, heavy suspensions of known content of atabrine-susceptible and -resistant cells were cytolyzed by repeated freezing and thawing, or by being incubated in the presence of 0.5 M glycine at 37 C for a period of 20 hours (Cowles, 1947). Cell-free opalescent enzyme preparations obtained by high-speed centrifugation following the use of either method were found to have lost their glucose dehydrogenase activity but to have retained those for hexose diphosphate and ethyl alcohol. These preparations were studied with respect to their susceptibility to the action of the drugs and for the activating effect of added riboflavin. Hexose diphosphate was used as the hydrogen donator in these experiments. The results are given in tables 1 and 2.

The results presented in table 1 show that, as with intact cells, the activity of cell-free preparations from drug-susceptible cells is inhibited by atabrine, acriflavine, and optochin. Propamidine has no inhibitory action on the hexose diphosphate dehydrogenase activity of the cell-free enzyme systems. The intact cells described in the preceding article were also not inhibited by propamidine. In contrast to the inhibition of the cell-free preparations from drug-susceptible cells, the activity of the corresponding preparations from the atabrine-resistant cells is little affected by atabrine and acriflavine. The inhibition exhibited by optochin is in harmony with the fact that atabrine and optochin resistances are not interrelated. Also, it can be seen from the table that the cell-free preparation from the resistant cells is less active than that of the susceptible cells. This can be correlated with the data previously obtained with intact cells.

The foregoing facts show that the behavior with respect to the drugs of the cell-free enzyme preparations from either kind of cells is comparable to those of the corresponding intact cells. It is therefore reasonable to state that the resistance behavior and decreased dehydrogenase activities of the drug-resistant pneumococcal cells are not due to an alteration of their cell wall permeability.

Response of cell-free enzyme systems to riboflavin. The results presented in table 2 deal with the response of the cell-free enzyme preparations to added riboflavin. It can be seen that the activity of the system containing, for example, 0.6 mg of autolyzed drug-susceptible cells undergoes an acceleration from 132

minutes of methylene blue reduction time for the control system to 75 minutes in the presence of 200 μg of added riboflavin (43 minutes with 400 μg of riboflavin).

TABLE 1

Drug inhibition of hexose diphosphate dehydrogenase activity of cell-free enzyme preparations of atabrine-susceptible and resistant pneumococci, type I

DRUG	CONCENTRATION OF DRUG	METHYLENE BLUE REDUCTION TIME (MINUTES)			
		Susceptible		Resistant	
		mg autolyzed cells used*			
		0.9	1.2	1.2	1.5
None	—	38	19	64	28
Propamidine	1:4,000	40	20	60	26
	1:1,000	48	18	64	28
Atabrine	1:10,000	80	39	70	30
	1:2,500	112	61	89	37
Acriflavine	1:40,000	62	29	65	32
	1:10,000	79	45	69	36
Optochin	1:8,000	71	31	73	35
	1:2,000	96	47	120	45

The system contained 0.004 M hexose diphosphate.

* Mg of autolyzed cells determined by subtracting turbidity values of bacterial suspension after autolysis from turbidity values of original suspensions.

TABLE 2

Effect of riboflavin on hexose diphosphate dehydrogenase activity of cell-free autolyzates of susceptible and atabrine-resistant pneumococci, type I

RIBOFLAVIN (μg/ml)	METHYLENE BLUE REDUCTION TIME (MINUTES)				
	Susceptible		Resistant		
	mg of autolyzed cells used*				
	0.6	0.9	0.6	0.9	1.2
None	132	40	>180	126	62
40	130	39	>180	125	61
100	99	37	>180	112	53
200	75	38	130	69	45
400	43	28	70	40	30

* See footnote, table 1.

Similarly, before the addition of riboflavin, under the same conditions, the control system containing 0.6 mg of autolyzed atabrine-resistant cells shows a reduction

time greater than 3 hours (overnight period). In the presence of 200 μg of ribo-flavin per ml the reduction time was 130 minutes, or an activity equaling that of the control made up of the drug-susceptible cell-free enzyme system. With 400 μg of riboflavin, the reduction time with the resistant cell system is 70 minutes, or an activity greater than that of the control made up of the drug-susceptible enzyme preparation. These facts demonstrate that the impaired dehydrogenase activities of drug-resistant cell-free enzyme systems are reactivable by the addition of riboflavin.

Inactivating effect of the reduction of the number of resistant cells in a system on the dehydrogenase activity. The data presented in table 3 show that a 2-fold dilution of drug-susceptible cells, as would be expected, causes a proportional increase in the methylene blue reduction time. This effect applies invariably to glucose, hexose diphosphate, and ethyl alcohol dehydrogenases. When the drug-resistant cells are similarly diluted, this relationship fails to hold. In the case of acriflavine- and optochin-resistant cells a twofold dilution causes at least a 3- to 4-fold increase in reduction time with glucose, and increases up to infinity in the case of atabrine- and propamidine-resistant cells. Similarly, with hexose diphos-phate and ethyl alcohol, a 2-fold dilution of the resistant cells increases their reduction time to infinity. The differences in activity of the susceptible and resistant cells are here emphasized by the fact that, in several instances, 0.2 mg of resistant cells fail to show any activity, whereas the same weight of drug-susceptible cells shows a high activity with all three substrates tested.

Restorative effect of riboflavin on the inactivated systems. The results presented in table 3 also show that the response to added riboflavin of the initially inactive systems, and those which were inactivated disproportionately by twofold dilution of the resistant cell suspensions, are remarkable. For example, 0.1 mg of atabrine-resistant cells in the presence of glucose were initially inactive. In the presence of 400 μg of riboflavin per ml, the system showed an activity (18 minutes of reduction time) equal to that of the reaction system containing 0.2 mg of the susceptible cells. Even 0.2 mg of the atabrine-resistant cells were inactive at the start in the presence of hexose diphosphate and ethyl alcohol. With 400 μg of riboflavin both systems showed, respectively, 67 and 47 minutes of reduction time. In this respect, the response by the ethyl alcohol dehydrogenase system was much more prompt, showing marked reactivation with 100 μg of riboflavin. Since, in the systems containing different weights of the susceptible parent cells, the activity is proportional to the number of cells, the activating effect of the added 400 μg of riboflavin on these systems is independent of dilution. For example, the glucose dehydrogenase activities of 0.2 mg and 0.1 mg of drug-susceptible parent cells correspond, respectively, to 7 and 16 minutes of reduction time. With 400 μg of riboflavin, the activity of the 0.1 mg of cells corresponded to 10 minutes of reduction time. Such a general stimulating effect by riboflavin is observed with all three substrates.

The data with sulfonamide are given only with glycerol, since only toward this substrate did the resistant organism show decreased dehydrogenase activity. As can be seen, there is only a minor accelerative effect by riboflavin on the decreased glycerol activity of the resistant cells. The effect of riboflavin on the decreased

activity of the optochin-resistant organisms is no greater than the stimulatory effect on the susceptible organisms.

Nicotinamide and thiamine were also tried in equimolar concentrations and were found to have no effect on the activity of any of the organisms.

TABLE 3

Effect of riboflavin on the dehydrogenase activity of susceptible and resistant type I pneumococci

ORGANISM	RIBOFLAVIN (μg/ML.)	GLUCOSE (0.001 M)		HDP (0.004 M)		ETHYL ALCOHOL (0.1 M)		GLYCEROL (0.1 M)
		mg of organisms used						
		0.2	0.1	0.2	0.1	0.2	0.1	0.5
		Reduction time in minutes						
Parent (susceptible)	None	7	16	13	21	7	15	30
	100	6	14	13	21	6	14	28
	200	6	12	12	19	6	13	26
	400	5	10	11	13	4	11	24
Acriflavine-resistant	None	9	34	83	∞	22	75	
	100	8	25	43	58	19	38	
	200	8	22	29	58	18	33	
	400	8	20	18	56	14	31	
Atabrine-resistant	None	20	∞	∞	∞	∞	∞	
	100	18	46	>300	∞	67	∞	
	200	17	40	>300	∞	58	113	
	400	13	18	67	75	47	80	
Propamidine-resistant	None	38	∞	43	∞	96	∞	
	100	37	∞	40	∞	69	∞	
	200	36	∞	38	∞	60	120	
	400	24	64	28	∞	32	44	
Optochin-resistant	None	53	162	∞	∞	>240	∞	
	100	50	93	>240	∞	>240	∞	
	200	37	80		∞	—	∞	
	400	34	73	153	>240	167	∞	
Sulfonamide-resistant	None	Same as susceptible						110
	100							110
	200							90
	400							78

The foregoing results show clearly that the drug-susceptible cells do not undergo any loss of activity per weight on dilution. In contrast, the resistant cells undergo a disproportionate or a complete inactivation on twofold dilution. The latter effect is reversible when the systems are supplemented with an excess of riboflavin. The implications of these effects will be considered in the discussion.

Reversible inactivation of the dehydrogenase activity by various temperatures. The data presented in table 4 show that keeping the suspensions of drug-susceptible types I and II pneumococcal cells for 2 hours at various temperatures in the absence of a substrate results in a measurable degree of inactivation. It can be seen, however, that, at the most, only a twofold increase in reduction time is experienced by the susceptible cells kept for 2 hours at 37 C. Under the same conditions, the atabrine-resistant type II cells experience a sixfold loss of activity. In contrast, type I drug-resistant cells after 2 hours' incubation at 37 C lose their activity completely. In this respect, type I atabrine-resistant cells, as would be

TABLE 4

Effect of exposure to temperature changes on the glucose (0.001 m) dehydrogenase activity of atabrine-susceptible and -resistant pneumococci; effect of riboflavin on decreased activity after exposure

ORGANISM (0.2 MG)	RIBOFLAVIN (µg/ML)	METHYLENE BLUE REDUCTION TIME (MINUTES)				
		Initial	After 2 hours at			
			4 C	22 C	37 C	44 C
Type II Parent (susceptible)	None	5	7	8	12	∞
Type II Atabrine-resistant	None	10	18	28	61	∞
Type I Parent (susceptible)	None	7	8	14	21	∞
	100		8	13	19	∞
	200		6	12	14	∞
	400		5	9	11	88
Type I Atabrine-resistant	None	12	16	52	∞	∞
	100		13	32	73	∞
	200		9	23	60	∞
	400		8	17	39	105

expected on the basis of a greater degree of resistance (see introduction), are more labile than type II atabrine-resistant cells.

Restoration of the lost activity of type I pneumococcal cells by added riboflavin. The results presented in table 4 also show that the slight inactivation that the drug-susceptible cells had undergone during incubation at 37 C can be nearly completely abolished by 400 µg of riboflavin. It is remarkable, however, that the drug-resistant cells which were completely inactivated at a temperature of 37 C are also reactivable. In the latter case, the infinitely long reduction time was reduced to 73, 60, and 39 minutes with 100, 200, and 400 µg of riboflavin, respectively. These results show that the inactivating effect of the 37 C temperature on the resistant cells is definitely more pronounced. It is significant that this inactivation is also reversible by the presence of an added excess of riboflavin.

Riboflavin contents of drug-susceptible and drug-resistant pneumococci. For an understanding of the mechanism of decreased dehydrogenase activities of the resistant cells and the accelerative effect of added riboflavin on these impaired systems, it was deemed necessary to obtain quantitative data regarding the riboflavin contents of these cells. The cells analyzed for this purpose were prepared from cultures which were grown for 18 hours in glucose broth. The cells were then thrown down in an angle centrifuge at 3,000 rpm. The cell sediments were washed once with distilled water, followed by washing with ice-cold acetone and ether free from fluorescent substances. The cells thus prepared were free from salts, as was indicated by negative tests for chloride and phosphate in the

TABLE 5

Riboflavin content of susceptible parent and drug-resistant pneumococci

ORGANISM	RIBOFLAVIN (μg/G) DRY CELLS
Type I—Mucoid parent (susceptible)........	63.69
Mucoid atabrine-resistant.	59.58
Mucoid acriflavine-resistant	65.04
Mucoid propamidine-resistant.....	69.60
Mucoid sulfathiazole-resistant . . .	59.34
Mucoid optochin-resistant.	42.11
Type II—Mucoid parent (susceptible)*.	39.21
Mucoid atabrine-resistant	40.46
Type III—Mucoid parent (susceptible)*.	83.92
Mucoid atabrine-resistant.....	57.83

Nicotinic acid and its derivatives: Type I atabrine-susceptible and atabrine-resistant cells were analyzed and found to contain, respectively, 410.3 μg and 432.1 μg of nicotinic acid and its derivatives per g of cells. The chemical method recommended by Perlzweig (1947) was used in these analyses.

* The rough organisms derived from types II and III, without exposure to any drug, were also analyzed and found to contain, respectively, 51.3 μg and 54.22 μg of riboflavin per g of cells.

supernatants of the aqueous suspensions. Samples of 250 mg of dry cells brought to constant weight were analyzed for riboflavin according to the fluorometric method of Arnold (1945). Analysis of yeast cells of known riboflavin content[a] indicated that washing the wet cell sediments with ice-cold acetone and ether does not extract riboflavin. Acetone and ether extracts concentrated to dryness and taken up in water failed to show measurable fluorescence.

The results presented in table 5 show that, with the exception of type I optochin-resistant cells, the drug-susceptible and drug-resistant type I and type II pneumococci contain the same amount of riboflavin per gram of dry cells. Riboflavin contents of type III atabrine-susceptible and atabrine-resistant

[a] Kindly supplied by Dr. E. M. Nelson and Dr. O. L. Kline of the U. S. Food and Drug Administration, Washington, D. C.

strains show considerable difference. This could not, however, be related to decreased dehydrogenase activity, since, of the three, type III resistant cells were least inclined to show decreased dehydrogenase activity.

As described in the footnote to table 5, type I atabrine-susceptible and atabrine-resistant pneumococci showed no appreciable difference in their content of nicotinic acid and its derivatives.

DISCUSSION

In growth experiments, resistance to drugs has often been assumed to be due to a physical change of the cell wall, such as a decreased permeability to the agents to which resistance was produced. Similarly, decreased dehydrogenase activity of the resistant cells might be thought to be due to decreased permeability to substrates. We have introduced a method of measuring drug resistance in the absence of growth, demonstrating that the enzymatic properties have undergone marked changes. By the use of this method we have clearly demonstrated that the reduced dehydrogenase activity of the resistant cells and resistance to drugs are demonstrable also with cell-free enzyme preparations. This finding shows that, after eliminating the permeability factor, we still can demonstrate the resistance of the enzymes to drugs as in intact cells. It is also shown that the response to added riboflavin is similar in degree with both cell-free enzyme preparations and intact cells. On the other hand, the cell-free enzyme preparations from drug-susceptible cells were still subject to inhibition as in intact cells. It is also shown that the response to added riboflavin is similar in degree with both cell-free enzyme preparations and intact cells.

In view of these observations, instead of an assumption of decreased permeability of the cell wall to drugs as the cause of resistance and decreased dehydrogenase activity, one must assume an alteration of intracellular enzymes. One must postulate that resistance to drugs and decreased dehydrogenase activities and an alteration in intracellular enzymes are phenomena which are causally interrelated. This view is supported by the fact that riboflavin antagonizes both the inhibition of growth and the inhibition of the enzyme by the drug. It would be difficult to explain those facts by assuming that the resistant cells had undergone, selectively, a decrease in permeability to atabrine and no change, in this respect, to riboflavin, which is a structural analogue of this drug.

On the contrary, the effects resulting from dilution and temperature and the elimination of these effects by added riboflavin suggest permeability of the cell walls of the resistant cells at least as great as that of the susceptible cells.

The results of growth experiments and dehydrogenase activity measurements with the susceptible cells show that riboflavin antagonizes the inhibitory action of the drugs. The present report shows that the impaired dehydrogenase activities, and inactivations by various treatments, are reactivable when an excess amount of riboflavin is added to the systems. These observations would seem to support the postulate (Sevag, 1946) that in the resistant cells the flavoprotein enzyme has undergone a specific change. The question must therefore

be asked concerning the nature of this alteration. To derive a satisfactory answer to this question the following facts should be considered:

(a) The dehydrogenase activity per weight of the drug-susceptible cells is not impaired on twofold or more dilution of the bacterial suspension. Resistant cells, in contrast to the susceptible ones, lose their activity more or less completely on dilution. These inactive systems are reactivated on the addition of an excess of riboflavin.

(b) The relatively insignificant effect of temperature, from 4 to 37 C, on the dehydrogenase activity of the drug-susceptible parent cells is contrasted by a marked loss of activity of the drug-resistant cells. This loss of activity is likewise reversible in the presence of added excess riboflavin.

(c) The absence of a difference in the riboflavin contents of the drug-susceptible and drug-resistant cells leaves unexplained the decreased dehydrogenase activities of the resistant cells. This conclusion is based on the assumption that the total riboflavin content of the cells represents active coenzyme, and that a deficiency does not exist in this component of the flavoprotein complex. The acceptance or rejection of this view of a deficiency in effective coenzyme must, of course, be based on the validity of this assumption.

Let us analyze the implications of the foregoing experimental findings. The amount of riboflavin required to reactivate the impaired dehydrogenase activities of the resistant cells is far greater than is normally required for the maintenance of normal physiological functions. In the presence of added excess riboflavin, the resistant cells show an activity which is greater than their normal activity, and very often approaches that of the drug-susceptible cells. Since riboflavin, per se, is believed to be incapable of acting as coenzyme but must first be converted to the coenzyme (alloxazine-adenine-dinucleotide or alloxazine mononucleotide), apparently a great excess of riboflavin is required for an immediately demonstrable activity. It would also seem possible that the resistant cells may possess systems so altered that coenzyme union with protein moieties requires an excess of riboflavin in order to force the reaction favoring the formation of a greater amount of active enzyme and to establish an effective equilibrium of flavoprotein $\rightleftharpoons$ protein $+$ coenzyme. At any rate, the riboflavin effects are specific to the extent that nicotinamide and thiamine fail to produce any effect whatsoever.

The considerations above may be restated by the use of the following reactions:

(1) Flavoprotein $\rightleftharpoons$ protein $+$ alloxazine mono- or dinucleotide
(2) Alloxazine dinucleotide $\rightleftharpoons$ alloxazine mononucleotide $+$ adenine nucleotide
(3) Alloxazine mononucleotide $\rightleftharpoons$ riboflavin $+$ H_3PO_4

Whenever reaction (1) is shifted to the left (due to the combination of the specific protein with the coenzyme), a greater activity will be manifested. This could best be verified if we could test the pneumococcal extracts with the complete coenzyme rather than with riboflavin. The activating effect of added excess riboflavin is to be attributed to the synthesis of additional coenzyme whereby the

reaction (3) is shifted to the left, which in turn shifts the reaction (2) to the left, etc.

The results with the resistant cells show clearly that, in contrast to the drug-susceptible cells, the foregoing three reactions are readily shifted under various conditions to the right, resulting in the additional loss of activity. Experimental data which can be marshaled at present in support of this concept are (a) a disproportionate loss of dehydrogenase activity on dilution of cells; and (b) a similar loss of activity when cells are subjected to temperatures higher than 4 C and up to 37 C. Increase of temperature appears to lead to dissociation of flavoprotein and loss of activity due to the diffusion of riboflavin resulting from the splitting of the dissociated coenzyme. Both of these treatments could cause increased dissociation of the altered protein and therefore further loss of activity. These effects are reversible by treatment with excess riboflavin, and are therefore not due to a denaturation of the protein moiety, unless we postulate that riboflavin is able to renaturate its specific protein partner in flavoprotein.

In view of the foregoing considerations, it would seem reasonable to assume that the protein moiety of the flavoprotein in the resistant cells has in some manner been altered, leading to decreased enzymatic activity. The fact that this activity can be raised to a level comparable to that of the drug-susceptible systems, would indicate that the impaired resistant systems possess inapparent potentialities which can be brought out under imposed conditions involving the participation of an excess amount of riboflavin. What may, however, appear to be difficult to interpret is the ineffectiveness of the complete quota of riboflavin which was found to be present in resistant cells in an amount equal to that of the highly active normal drug-susceptible cells. This fact may be interpreted to indicate that the link between the preformed riboflavin (or coenzyme) and the protein moiety of flavoprotein is in some manner impaired. This defect would appear to be due perhaps to an alteration of the protein moiety of flavoprotein arising from the circumstances underlying the development of resistance to a drug. It may also be possible that the flavoprotein of the resistant cells exists in an equilibrium state as follows: inactive impaired protein ⇌ normally active protein.

If the protein is so altered, resulting in decreased affinity with the coenzyme (higher dissociation constant), a decrease in activity would result. The experimental data cited above would seem to support this explanation. Under conditions imposed upon the system by excess riboflavin, the equilibrium apparently shifts to the left, thereby increasing the activity to full capacity.

Assuming from the data of riboflavin antagonism that the drug competes with the coenzyme for the flavoprotein, an alteration of flavoprotein leading to lowered combining power with the prosthetic group and thus to lowered activity might also be responsible for the reduced combining capacity with the drug, resulting in a lower susceptibility of the organism to the drug. This would seem to offer a reasonable explanation for the phenomenon of resistance to drugs by the pneumococcal cells.

Since the altered protein is inheritable in an altered state from generation to

generation of pneumococcal cells, the specific genetic factors of these cells responsible for the synthesis of the altered enzyme proteins could be assumed to have undergone specific changes. If our assumptions are justifiable, then it is reasonable to believe that the drugs are interfering simultaneously with the normal functions of the oxidative enzymes and of the genetic factors, or with the interdependence of the functions of the enzyme proteins and genetic factors resulting in the impaired enzyme activities of the drug-resistant cells. A study of the biochemistry of the nucleoprotein components of these cells would be a further reasonable approach to the broader understanding of the resistance phenomenon.

SUMMARY AND CONCLUSIONS

The riboflavin contents of drug-susceptible and drug-resistant cells have been determined in relation to the impaired dehydrogenase activities of drug-resistant cells. The effects of dilution and temperature on the dehydrogenase activities of both the drug-susceptible and drug-resistant cells were determined in relation to the possible alteration of the enzyme proteins.

Cell-free enzyme preparations made from either cells show dehydrogenase activity with hexose diphosphate and ethyl alcohol. Extracts from susceptible cells are subject to inhibition as in intact cells. Extracts from the drug-resistant cells are resistant to drugs as in intact cells; these extracts show impaired dehydrogenase activity as observed in intact cells. The impaired normal activities of the extracts from resistant cells are reactivated on addition of excess riboflavin as in intact cells. These findings show that the drug-resistant cells have not undergone decreased permeability to drugs, substrates, and riboflavin. In fact, the data suggest that resistant cells may manifest greater permeability to these agents.

Analysis for the riboflavin contents of both the drug-susceptible and drug-resistant cells shows no quantitative difference. The impaired dehydrogenase activities of the drug-resistant cells, therefore, do not appear to be due to a deficiency in riboflavin.

Dehydrogenase activity per weight of the susceptible cells does not undergo diminution on dilution; their activity is independent of dilution. In contrast, the resistant cells lose their activity on twofold or more dilution; the lost activities are recoverable on the addition of riboflavin. The effect of temperature, from 4 to 37 C, on the susceptible cells is negligible. In contrast, the drug-resistant cells undergo marked or complete loss of activity under these conditions. This activity is recoverable on the addition of riboflavin.

The absence of a difference in the riboflavin contents of drug-susceptible and drug-resistant cells, the loss of activity on dilution and on exposure to temperature, and the recoverability of the lost activities on treatment with excess riboflavin have been interpreted to indicate that the flavoproteins of the drug-resistant cells are subject to greater dissociation and thereby loss of activity. This is attributed to an alteration of the protein component of the enzymes associated with the development of resistance. The inheritability of these char-

acteristics in the absence of the drug permits the assumption that genetic factors have likewise undergone mutations.

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CIRCULIN, AN ANTIBIOTIC FROM A MEMBER OF THE BACILLUS CIRCULANS GROUP

I. Bacteriological Studies

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During the course of a search for new chemotherapeutic agents, a bacillus showing antibiotic properties was obtained from soil. The organism was isolated by plating dilutions of soil in nutrient agar seeded with *Escherichia coli* and fishing colonies which grew out surrounded by zones of inhibition. This culture, no. M-14, produces a substance[1] that is active against gram-positive and gram-negative bacteria and against numerous fungal species. It is also lytic for red blood cells.

The present paper is concerned with a description of the organism and with bacteriological studies conducted with the active substance. The antibiotic, which has been named circulin, is a polypeptide and has been obtained in crystalline form. The method of extraction and purification of the substance will be reported elsewhere (Howell, 1948).

Description of organism. The M-14 culture is composed of gram-variable, encapsulated, motile rods that produce oval terminal spores. The vegetative forms occur singly and have rounded ends. On nutrient agar the organism gives a very mucoid adherent growth in 18 hours at 37 C. In nutrient broth the growth is initiated much more slowly and a mucoid pellicle is formed. On potato slants the growth has a creamy tan color.

Acid without gas is produced from glucose, fructose, maltose, sucrose, galactose, raffinose, xylose, and dextrin. Arabinose, rhamnose, mannose, lactose, glycerol, inulin, and salicin are not fermented. The culture reduces methylene blue, which is reoxidized within four days. The Voges-Proskauer test is negative. Gelatine, casein, and starch are hydrolyzed. Nitrites are produced from nitrates.

According to Dr. Nathan R. Smith (1948), who very kindly studied our culture, it seems to be identical with *Bacillus krzemieniewski* (Smith, Gordon, and Clark, 1946), which has been tentatively placed in the *Bacillus circulans* complex as a mucoid variant. The M-14 culture differs from *Bacillus krzemieniewski* in that it is more strongly saccharolytic and in that growth is more slimy and occurs at 45 C.

Production of the antibiotic. The active substance is formed in both stationary and shaken cultures at 23 C and at 37 C. Growth of the culture and production

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[1] Since the completion of this work a preliminary report has appeared describing an antibiotic produced by a nonmucoid strain of *Bacillus circulans*. This substance differs from circulin in being water-soluble, relatively nontoxic, and more active against gram-negative than gram-positive organisms (Murray and Tetrault, 1948).

of the antibiotic proceed more slowly at the lower temperature. A good yield is obtained in media containing tryptone, protone, "phytone," "protolysate," "pepticase," "NZ-case," "amigen," or Difco peptone. The antibiotic is formed also in synthetic media containing alanine, asparagine, glutamic acid, or glycine. Glucose, sucrose, maltose, and glycerol are equally efficient sources of carbon. A slightly lower yield is obtained with lactose.

The antibiotic is produced routinely in a synthetic medium consisting of 0.5 per cent DL-alanine, 0.5 per cent monopotassium phosphate, 0.25 per cent magnesium citrate, 0.05 per cent magnesium sulfate, and 2.0 per cent glycerol in distilled water. The medium is adjusted to pH 7.2 and dispensed in 500-ml amounts to a depth of 1 ml in large flasks. After sterilization at 120 C for 15 minutes, the flasks are inoculated with 2 ml each of a spore suspension, prepared from a one-week-old culture on nutrient agar and incubated at 37 C. No evidence of growth is apparent until after 48 hours when a thin mucoid pellicle begins to form. At the end of 10 days the entire medium is a mucoid mass in which little or no sporulation of the organisms has occurred. At this time the medium is pH 7.0 and maximum titers are obtained, usually 300 *Escherichia coli* units and 100 hemolytic units per ml.

Method of assay. The active substance contained in the original harvest and in the purified fractions diffuses poorly on agar plates. Potency is measured by assaying serial dilutions of the antibiotic against broth cultures of the test organism. The unit of activity has been defined as the minimum amount of active substance that will completely inhibit the growth of *Escherichia coli* (VDRL 76) in 1 ml of nutrient broth for 24 hours. Since circulin is more soluble in water at pH 2.0 than at neutrality (Howell, 1948), dilutions are made in distilled water that has been adjusted to pH 2.0 with normal hydrochloric acid. The samples are diluted 1:100, 1:300, and 1:500, and these solutions are further diluted twofold serially. One ml of each dilution is mixed with 1 ml of a culture suspension that is prepared by adding one drop of saturated sodium hydroxide and 1 ml of an overnight *E. coli* culture to 100 ml of nutrient broth. The pH of the resulting mixtures is 7.0. Test cultures are incubated in a water bath at 37 C and are read at the end of 24 hours. No change in the reading occurs on longer incubation.

Circulin is hemolytic for human, rabbit, sheep, and mouse red blood cells. The hemolytic titer of the samples is assayed by testing against a standard suspension of 2 per cent washed and packed rabbit erythrocytes. Dilutions of the antibiotic in 0.85 per cent saline adjusted to pH 2.0 with normal hydrochloric acid are dispensed in 1-ml amounts in assay tubes. One ml of 0.05 molar phosphate buffer, pH 7.0, is added to each tube, followed by 0.5 ml of rabbit red cells suspended in physiological saline. Tests are read after incubation for 1 hour in a water bath at 37 C. Maximum hemolysis usually occurs within 15 minutes.

Activity and stability of crystalline preparations. Assays of crystalline circulin indicate that the antimicrobial activity of the original culture broth has been concentrated to a greater extent that the hemolytic activity. One ml of the original medium contains 300 *E. coli* units and 100 hemolytic units, whereas the amount

of crystalline material that contains 300 *E. coli* units possesses only 24 hemolytic units. Some preparations of purified circulin have assayed 0.3 *E. coli* units and 0.0274 hemolytic units per microgram of crystalline material. Or, to express this relationship in another manner, one *E. coli* unit is equivalent to 3 micrograms and one hemolytic unit is equivalent to 36.5 micrograms.

The antibiotic is filterable through a Zeitz filter with no loss of activity. Crystalline preparations in dilute hydrochloric acid solution may be stored for a period of 2 years, or probably longer, at 4 C with no loss of activity. The antibiotic withstands autoclaving for 30 minutes in acid or neutral solution. Autoclaving in alkaline solution results in a decline in titer with a greater loss of activity against *E. coli* than against red blood cells. The results of a typical experiment are summarized in table 1.

Antimicrobial spectrum of circulin. The activity of crystalline circulin against a number of bacterial and fungal species expressed in micrograms per ml of test medium is shown in table 2. In assays with all bacterial cultures, with the exception of *Mycobacterium tuberculosis*, a 1 per cent suspension of an overnight culture in broth was used. Tests were read after incubation for 24 hours at 37 C. In assays with *M. tuberculosis* the inoculum was 0.1 ml of a 7-day culture in Dubos medium. These tests were incubated for 1 month at 37 C. Fungal species were tested in broth containing 2 per cent peptone and 4 per cent maltose dispensed in 50-ml amounts in small flasks, each flask being inoculated with 0.5 ml of a suspension of the organism. Tests were read after 2 weeks' incubation at room temperature.

Bactericidal action of circulin. Circulin is bactericidal in its action as well as bacteriostatic. Table 3 shows the results of a typical experiment in which 5 units apparently killed an inoculum of approximately 300,000,000 *E. coli* in 24 hours. Dilutions were made of a purified fraction so that 25, 10, 5, 2.5, 1.25, or 0.625 units were contained in 1 ml. These dilutions were mixed with 1 ml of *E. coli* culture in broth and incubated for 24 hours at 37 C. At the end of this period the tubes showing no visible growth were centrifuged for 1 hour in an angle-head centrifuge in order to sediment the original inoculum of organisms. By using aseptic technique, the organisms in each tube were washed in 2 ml saline, resedimented, and resuspended in 2 ml broth. The organisms which had been exposed to as little as 5 units of circulin failed to grow during incubation for 1 week at 37 C.

Effect of blood serum upon activity. The presence of a final concentration of 25 per cent blood serum in the assay system has no appreciable effect upon the activity of circulin against *E. coli* or red blood cells. The results of a typical experiment are shown in table 4. Twofold serial dilutions of a crystalline preparation were made in duplicate in distilled water or 0.05 molar phosphate buffer, pH 7.0. In *E. coli* assays, 1 ml of nutrient broth or 50 per cent serum broth containing approximately 10,000 organisms per ml was added to each tube containing 1 ml of the antibiotic in distilled water. To tubes containing 1 ml of the antibiotic in buffer solution, 0.5 ml of buffer or serum was added, followed by 0.5 ml of a 2 per cent suspension of rabbit blood cells in physiological saline. Test cultures were incubated for 24 hours at 37 C.

TABLE 1

Effect of pH on heat stability of circulin

SOLUTION OF CRYSTALS pH	DILUTIONS SHOWING HEMOLYTIC ACTIVITY OR COMPLETELY INHIBITING GROWTH OF E. COLI			
	After 24 hours at room temperature		After 30 minutes in autoclave	
	Hemolytic activity	E. coli inhibition	Hemolytic activity	E. coli inhibition
2.0	1:32	1:512	1:32	1:512
7.0	1:32	1:512	1:32	1:512
8.0	1:32	1:512	1:16	1:128
9.0	1:32	1:512	1:4	1:8

TABLE 2

Amount of crystalline circulin completely inhibiting growth of test organisms

TEST ORGANISMS	TEST BROTH	µg/ML
Staphylococcus aureus (FDA 209 P)	Nutrient	1.5
Diplococcus pneumoniae, type 1	Beef heart	1.5
Streptococcus pyogenes (C-203)	Beef-heart	1.5
Escherichia coli (VDRL 76)	Nutrient	3.0
Escherichia communior (ATCC 7011)	"	1.5
Eberthella typhosa (ATTC 7251)	"	3.0
Salmonella paratyphi (ATTC 9150)	"	3.0
Salmonella schottmuelleri (ATTC 9149)	"	3.0
Klebsiella pneumoniae, type A (ATCC 8045)	"	1.5
Klebsiella pneumoniae, type B (ATCC 8052)	"	3.0
Salmonella typhimurium (ATCC 9148)	"	4.5
Shigella dysenteriae (ATCC 8708)	"	1.8
Pseudomonas aeruginosa	"	10.5
Bacillus subtilis (ATCC 6633)	"	0.75
Bacillus mycoides (ATCC 6462)	"	3.0
Clostridium perfringens (AMS)	Thioglycollate	3.0
Clostridium histolyticum (AMS)	"	1.5
Clostridium fallax (AMS)	"	3.0
Clostridium tertium (AMS)	"	7.5
Mycobacterium tuberculosis (H37Rv)	Dubos	4.5
Monilia albicans	Sabouraud's	3.0
Trichophyton rubrum	"	3.0
Epidermophyton floccosum	"	1.5
Blastomyces dermatitidis	"	1.5
Sporotrichum schencki	"	1.5
Actinomyces graminis	"	1.5
Histoplasma capsulatum	"	1.5
Microsporum audouini	"	1.5
Microsporum lanosum	"	1.5

Toxicity of circulin. Circulin is extremely toxic for mice. The LD_{50} dose by intraperitoneal injection is 0.3 mg. This dose contains 100 *E. coli* units and approximately 8 hemolytic units. Somewhat larger amounts may be adminis-

tered subcutaneously or, in divided doses, intraperitoneally. The excretion of the antibiotic and the chemotherapeutic action have not been determined.

Differentiation of circulin from antibiotics with similar action. Although differing in chemical (Howell, 1948) and other properties, circulin is somewhat similar in its antimicrobial spectrum to gramicidin "S," streptothricin, and tyrocidine. These antibiotics are toxic and are active against both gram-positive and gram-negative bacteria (Waksman, 1947).

Both gramicidin "S" (Manevich and Pitskhelauri, 1944) and circulin are hemolytic. Circulin is active against M. tuberculosis and fungi. Gramicidin "S" is inactive against M. tuberculosis (Frieden, 1945), and a search of the literature revealed no statement as to the activity of this antibiotic against fungi.

TABLE 3

Bactericidal action of circulin against E. coli

CIRCULIN, UNITS	INOCULUM OF E. COLI	GROWTH IN 4 HOURS	ORGANISMS WASHED IN SALINE AND RESUSPENDED IN BROTH GROWTH IN 7 DAYS
25	300,000,000	—	—
10	300,000,000	—	—
5	300,000,000	—	—
2.5	300,000,000	—	+
1.25	300,000,000	—	+
0.625	300,000,000	+	
0	300,000,000	+	

TABLE 4

Effect of 25 per cent blood serum upon the activity of circulin

CRYSTALLINE CIRCULIN	DILUTION HEMOLYZING RED BLOOD CELLS	DILUTION COMPLETELY INHIBITING GROWTH OF E. COLI
No serum in assay system......................	1:64	1:1,024
25 per cent serum in assay system....	1:64	1:1,024

Streptothricin may be distinguished from circulin by its inactivity against Bacillus mycoides and against clostridial species (Waksman and Woodruff, 1942). Both tyrocidine (Dubos and Hotchkiss, 1942) and circulin have strong bactericidal properties and immediate hemolytic action. Tyrocidine differs from circulin in possessing no activity against M. tuberculosis (Frieden, 1945; Waksman, 1947), in the loss of activity against gram-negative species in the presence of peptones (Dubos and Hotchkiss, 1942), and in the loss of activity against red blood cells in the presence of serum (Mann, Heilman, and Herrell, 1943).

DISCUSSION AND SUMMARY

A new antibiotic, circulin, has been obtained from broth cultures of a mucoid variant of Bacillus circulans. This substance is active against both gram-

positive and gram-negative bacteria and against fungi.   It is bactericidal as well as bacteriostatic, and the activity is not appreciably reduced in the presence of blood serum.   Differences have been pointed out in the spectrum of circulin and that of other antibiotics which have similar action.

Circulin is hemolytic and extremely toxic.   Although unsuitable for parenteral administration, limited trials indicate that it has possible value in the treatment of bacterial and fungal infections of the skin.

In an effort to reduce the toxicity of circulin, attempts have been made to reduce the hemolytic activity.   Treatment with formalin, which reduced the hemolytic action of gramicidin (Lewis et al., 1945), was unsuccessful.   Since approximately three-fourths of the hemolytic activity of the original culture broth is lost during the purification of circulin, it seems probable that two different substances are produced by the M-14 culture.   It is possible that the hemolytic activity of circulin is caused by some of the hemolytic substance remaining as an impurity in the crystalline antibacterial antibiotic.   Findings similar to this were reported by Waksman and Geiger (1944) in the case of crystalline fumigacin from which gliotoxin was removed.   Further attempts will be made to reduce the toxicity of circulin.

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MORPHOLOGIC CHARACTERISTICS OF CERTAIN CULTURED STRAINS OF ORAL SPIROCHETES AND TREPONEMA PALLIDUM AS REVEALED BY THE ELECTRON MICROSCOPE

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Knowledge of spirochetal morphology has been gained for the most part by systematic studies of material from infected hosts and in certain instances from pure cultures maintained in the laboratory on artificial culture media. In this regard the hanging drop preparation, the stained smear or section, and dark-field illumination of wet preparations have been used to advantage. The last method has been more universally employed by the exacting investigator to demonstrate the fine structural differences that exist between the various types of spirochetes.

The members of the genera *Treponema* and *Borrelia* present morphologic and structural features that have been the subject of considerable speculation and controversy for many years. In this laboratory the electron microscope has been used to demostrate some of these controversial structures, and the present paper describes certain results thus far obtained.

Eight strains of spirochetes have been used. Two of these were strains of the small oral treponemes identified as FM and MRB; four were strains of *Borrelia vincentii*, designated as N9, N19, N37, and CON; and two were cultured strains of *Treponema pallidum* denoted as Nog and Ni (the Noguchi and Nichols strains). The small oral treponemes were morphologically and culturally similar in all details and representative of our collection of this type of spirochete. Under dark-field optical illumination, they showed uniform and closely spaced spirals and had long tapered ends. They were not double contoured. The four strains of *B. vincentii* were morphologically and culturally similar to one another and were approximately one and one-half times the size of the smaller oral treponemes. By dark-field examination these spirochetes had large, uniform, and widely spaced coils. The extremities were abruptly tapered and the organisms were not double contoured. The strains of *T. pallidum* were culturally similar to one another; the organisms had uniform and closely spaced spirals and long tapered ends and were not double contoured. In this they resembled the oral treponemes, but were on the average about one and one-half times as long.

All spirochetes were grown in a clear liquid medium which is a modification of a "hormone" agar described by Huntoon (1918). This modified broth is essen-

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tially a veal heart infusion in which "thio-peptone" is substituted for "bacto-peptone" and agar omitted. Prior to use, the medium was filtered through a Berkefeld "N" filter to remove particulate matter. At the time of use, 100-ml portions of the basic medium were enriched with 10 ml of a 1 per cent solution of glutathione and 10 ml of ascitic fluid. Volumetric 25-ml flasks, filled to within 2 ml of the top with this finished medium, were inoculated with spirochetes. The flasks were rubber-stoppered and the cultures incubated at 37 C. At the expiration of the desired incubation period, the cultures were centrifuged and the sedimented organisms washed and recentrifuged five to seven times in distilled water to remove salts, soluble proteins, and particulate matter. This treatment has been found necessary in order to provide sufficiently clean preparations for electron microscopy. After the last washing the spirochetes were suspended in 0.1 per cent formaldehyde in distilled water and placed in the refrigerator over-night.

The stock suspensions of spirochetes were diluted to arbitrary concentrations and microdrops dried on the usual collodion-covered specimen screens. Approximately 300 preparations were made from more than 30 separate cultures. These were examined with an RCA type EMU microscope after being "shadowed" by oblique evaporation of 40 mg chromium from a filament placed 18 cm behind and 3 cm above the specimens.

The following typical electron micrographs demonstrate the morphologic characteristics, fine structures, and "granules" typical of these strains of organisms.

General morphology. Figures 1, 2, 3, and 4 are micrographs of the small oral treponemes, *Borrelia vincentii*, and the Nichols strain of *Treponema pallidum*. The oral treponemes of figures 1 and 2 represent the larger organisms of this strain, and exhibit shallow, closely spaced spirals. A short form of *B. vincentii* (figure 3) was selected in order that the entire organism might be included within a single microscopic field. Its spirals are larger and deeper and its ends are abruptly tapered. In addition, one end possesses a hook-shaped structure that is often observed in shadowed preparations but has not been observed during dark-field examination of living organisms. Spirochetes of the Nichols strain of *Treponema pallidum* (figure 4) are approximately as wide as the small oral treponemes and their ends are gradually tapered. The organism in the upper part of figure 4 shows spirals with about the same amplitude as those of the small oral spirochetes.

Spirochetes in most of the preparations for electron microscopy appear straighter than the corresponding living organisms seen by dark-field illumination. Such changes have been observed in stained smears of these organisms and they have been shown to be a result of drying in air and heat fixation (Hampp, 1945).

Surface structure. The external appearance of all types of spirochetes studied is strikingly similar (figures 1, 2, 3, 4). Definite cell membranes have not been observed; if they exist they must be tightly adherent to the organisms. Most surfaces are not smooth and their irregularities, as indicated by the shadows cast, may be an expression of uneven distribution of the protoplasm.

Figure 1. Small oral treponeme (strain FM). × 11,000.
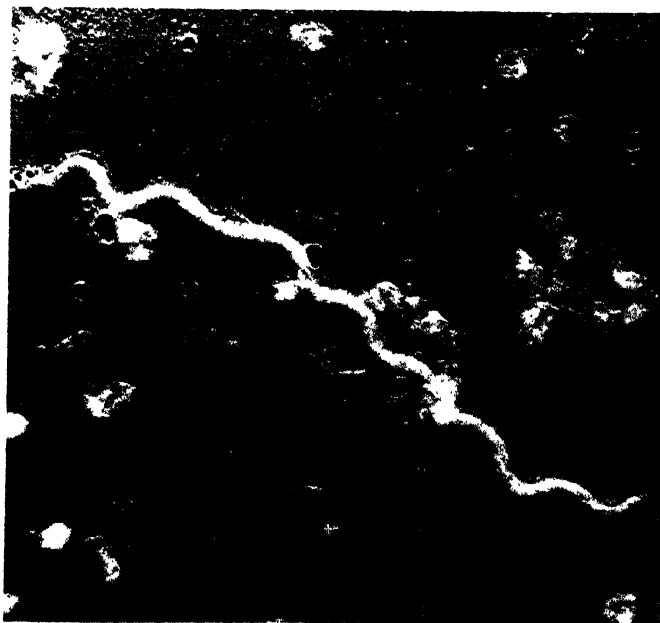
Figure 2. Small oral treponeme (strain MRB). × 11,000.
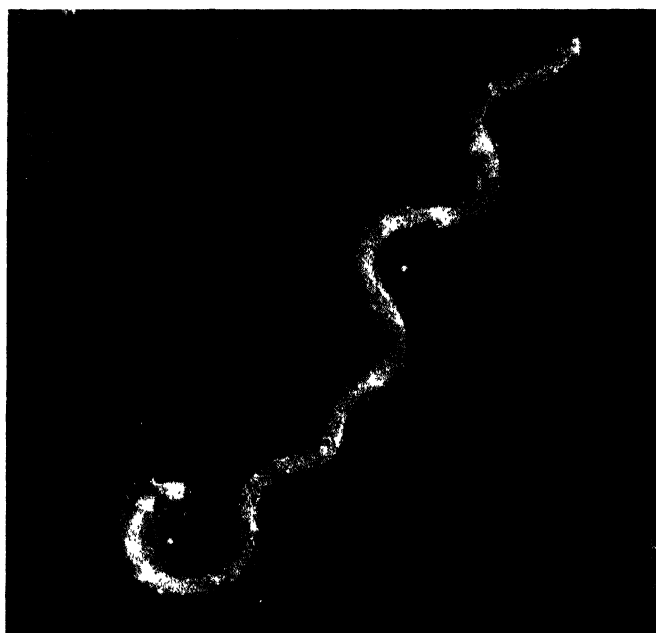
Figure 3. Borrelia vincentii (strain N19)   × 11,000.

Figure 4. Treponema pallidum (Nichols strain).   × 11,000.
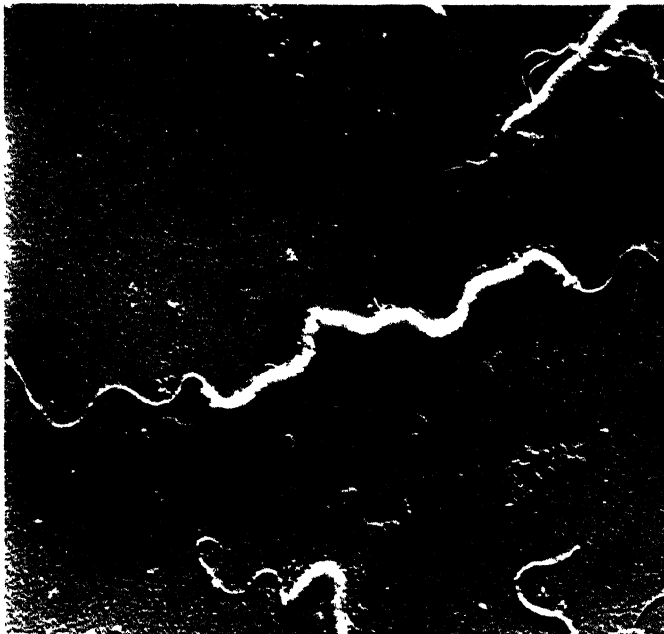
Figure 5. Small oral treponeme (strain MRB)   × 11,000.
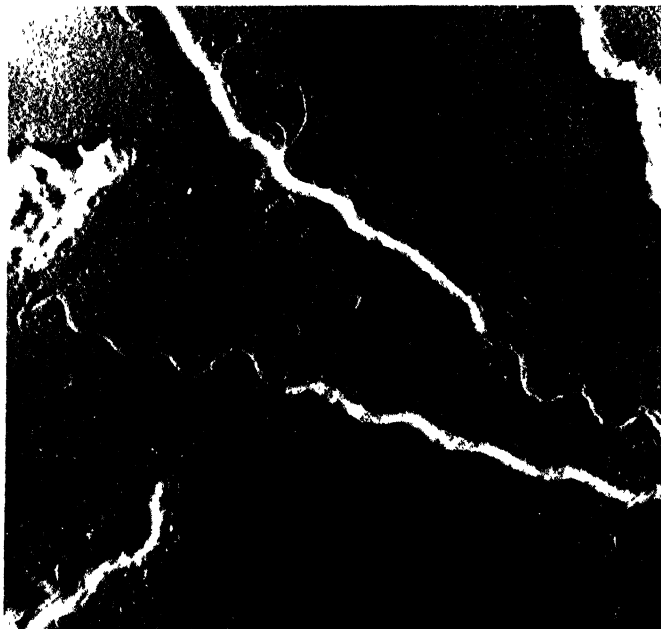
Figure 6. Small oral treponeme (strain FM).   × 11,000.

Filamentous structures. Terminal filaments have been a constant finding by dark-field examination of young, rapidly proliferating cultures of spirochetes. A large percentage of individual cells have shown these appendages at one or both ends. They have usually been coiled in the same manner as the spirochete, and have appeared to be single fibers. The appearance of these filaments under the electron microscope is illustrated in figures 5, 6, and 7. Some are short; others are longer than the spirochetes themselves. At the high magnifications of the electron micrographs it is clear that terminal filaments are not single fibers, but consist of intertwined fine strands (figures 6 and 7). Smith (1932) has postulated that these structures represent a continuation of the cell wall or "periplast," but no evidence has been found to support this assumption.

In addition to the terminal filaments there are many other flagellar processes attached to the spirochetes of all strains studied. The presence of such flagella has also been observed by other investigators using the electron microscope (Wile *et al.*, 1942; Morton and Anderson, 1942; Wile and Kearney, 1943; Mudd *et al.*, 1943). Evidently the previously held opinion (Bergey, 1939; Topley and Wilson, 1936) that spirochetes are devoid of flagella is incorrect. Flagella occur anywhere along the spirochetes. They are found singly and in tufts and are sometimes intertwined to form cords (figures 1 through 10). When granules are present, many flagella are often seen in association with them (figures 16, 20).

Internal granules. Internal granules have been frequently observed within the cytoplasm of spirochetes, both by dark-field illumination and by stained smears. They have often been seen in the aging cultures of the strains of oral spirochetes and *T. pallidum* (Hampp, 1946; Hampp, unpublished data), which have been used in the present investigation. Their typical appearance under the electron microscope is illustrated by figures 11 and 15. The granules produce irregularities in the surface of the organism, as evidenced by their shadows, and seemingly represent local accumulations of cytoplasm.

End granules. Another type of granule is occasionally seen that is different from either the internal granules or the external granules described below. It is small, occurs at the end of an organism, and lacks visible internal structural detail (figure 12). Mudd *et al.* (1943) have referred to this structure as an "end granule."

External granules. Periodic dark-field examination of spirochetal cultures indicates that about 2 to 3 weeks after inoculation another type of granule appears (Hampp, 1946); in the early stages of their formation they are intimately associated with the organisms and may occupy middle, subterminal, or terminal positions. Later they are "shed" and are found free in the culture medium.

Typical granules associated with spirochetal cells are shown in figures 13, 14, 15, 16, and 17. In figures 13 and 14 a portion of each organism is enclosed within a spherule. In the case of the middle granules of figures 16 and 17 there is a different relationship between these structures and the somatic substance of the organisms. In figure 16 an opaque region can be seen within the granule surrounded by a homogeneous substance, which is sharply delineated. A profusion of flagella are exhibited. Figure 17 demonstrates a granule, the content of which
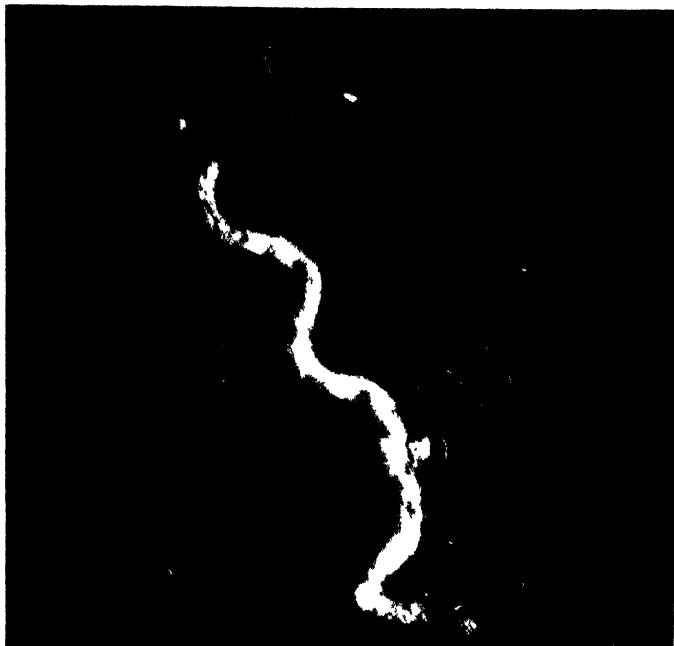
Figure 7. Borrelia vincentii (strain N37). × 15,000.
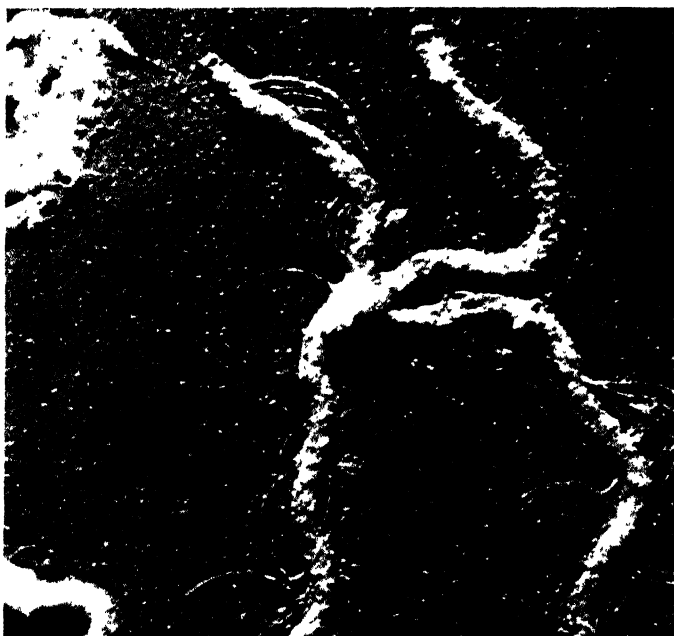
Figure 8. Borrelia vincentii (strain CON). × 14,000

Figure 9 *Borrelia vincentii* (strain CON). × 11,000.
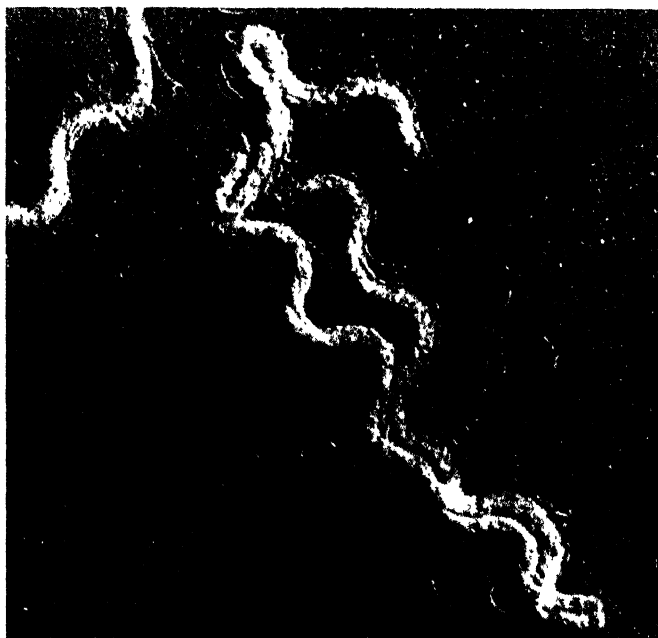
Figure 10. *Borrelia vincentii* (strain CON). × 11,000.

Figure 11. Borrelia vincentii (strain 19)   × 11,000.
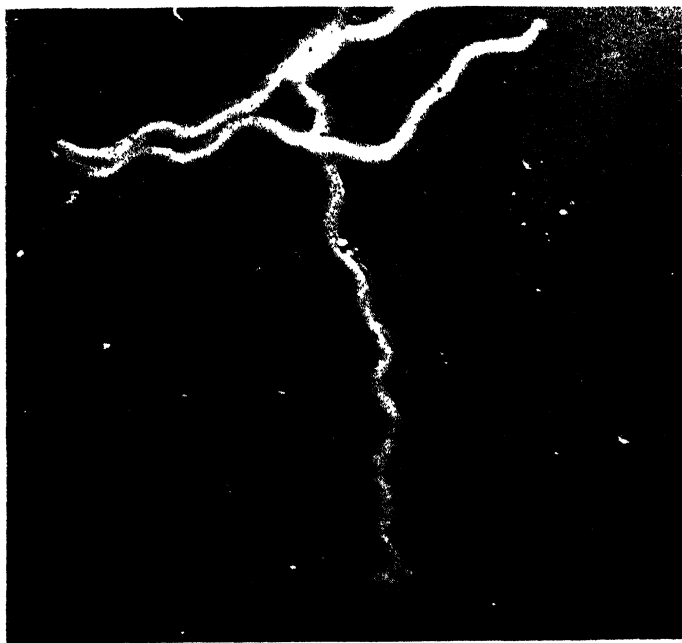
Figure 12. Borrelia vincentii (strain CON).   × 11,000.

Figure 13. Borrelia vincentii (strain N37)   × 11,000

Figure 14. Borrelia vincentii (strain N19).   × 11,000.
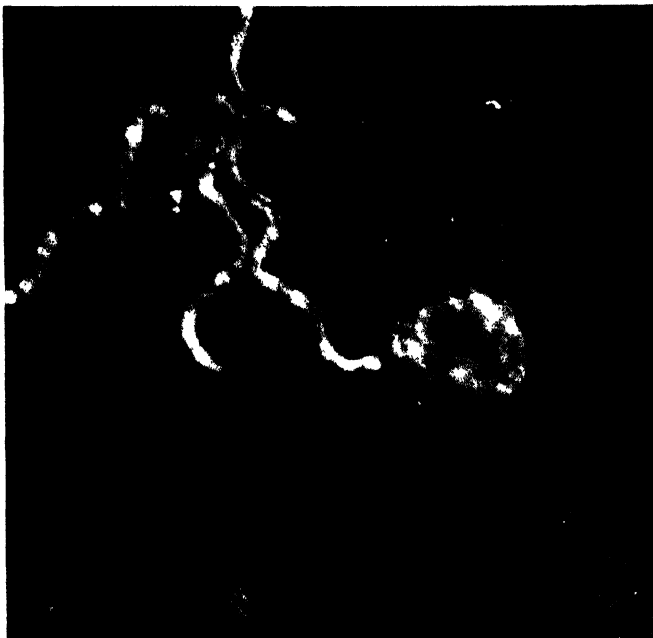
Figure 15. Borrelia vincentii (strain N19).   × 11,000.

Figure 16. Borrelia vincentii (strain N19).   × 11,000.
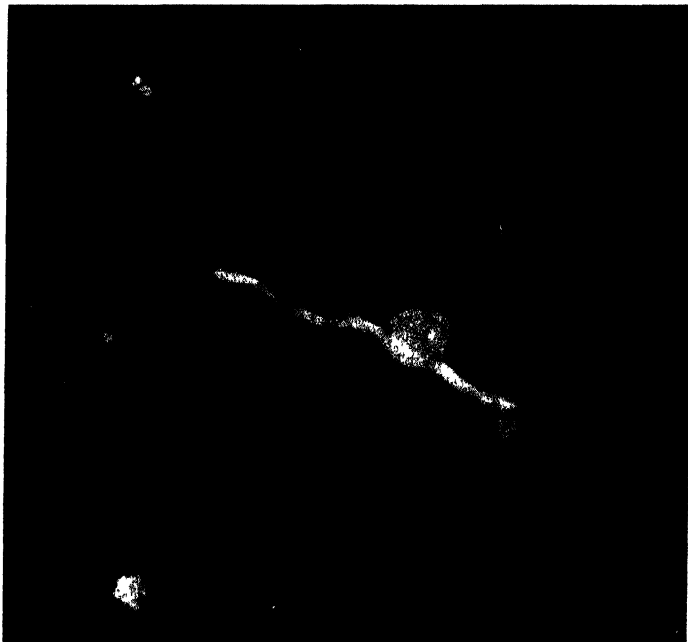
Figure 17 Borrelia vincentii (strain N37)   × 11,000.
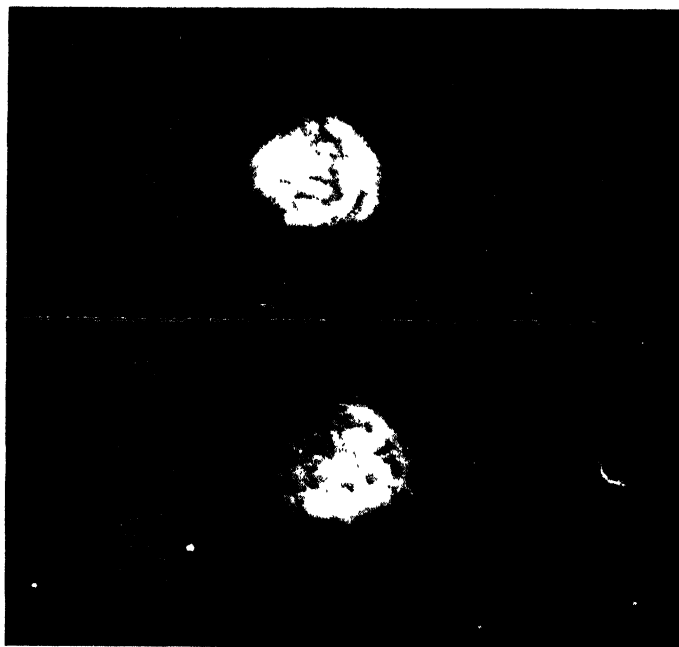
Figure 18. (Top) Borrelia vincentii (strain N37). × 11,000. (Bottom) Treponema pallidum (Nichols strain). × 11,000.

Figure 19. Borrelia vincentii (strain N9). × 11,000

Figure 20. Borrelia vincentii (strain N19). × 11,000.

is seemingly like the cytoplasm of the organism and blends with it.   None of the granules examined was attached to a spirochete by a stalk, as indicated by Mudd *et al.* (1943).   The content of the granule of figure 15 more nearly resembles that of free granules and may represent a more advanced stage in granule development.

Typical free granules, the end products of granule "shedding," are shown in figure 18.   They are roughly circular in outline and sharply bounded.   They consist for the most part of what appear to be short sections of spirochetes closely packed together.   The contents of these granules are probably responsible for the fine lacelike appearance and the bright white, highly refractile bodies described by Hampp (1946) under the dark-field microscope.

Examples of another type of free granule repeatedly observed are shown in figures 19 and 20.   These granules consist of tangled masses of spirochetes or spirochetal segments.

The significance of granules in the life history of the spirochetes is unknown but certain investigators have suggested that they may be germinative units (Balfour, 1911; Noguchi, 1911; Noguchi, 1917; Leishman, 1918; Mudd *et al.*, 1943; Hampp, 1946).   Others are undecided or hesitant in accepting this hypothesis (Fantham, 1916; Akatsu, 1917; Wenyon, 1926; Warthin and Olsen, 1930).   Topley and Wilson (1936) have indicated that they are probably particles of culture medium adhering to the sides of the spirochetes.   The electron micrographs demonstrate that this explanation is wrong, and that free granules are definitely a phase in the development of spirochetes.   Although it is not possible to determine from these micrographs that the granules are germinative units, their constant rhythmic occurrence in living cultures suggests this possibility.   Further support of this hypothesis is provided by the fact that cultures up to 31 months old, showing only refractile granules by dark-field examination, have invariably given normal growths on transfer to fresh medium (Hampp, 1946).

SUMMARY

Shadowed preparations of pure cultures of two strains of the small oral treponemes, four strains of *Borrelia vincentii*, and the Nichols and Noguchi cultured strains of *Treponema pallidum* have been studied with the electron microscope. Morphological characteristics, filamentous and flagellar appendages, and granules of various types have been described and illustrated.

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THE CONTROL OF SALMONELLA INFECTIONS IN COLONIES OF MICE[1]

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The need for a practical method of control of *Salmonella* infections in colonies of laboratory mice has long been emphasized both by research workers and by commercial breeders of mice. Although various methods have been suggested from time to time, such proposals have not resulted in any consistent long-term practical control of paratyphoid infection. Some of the proposed procedures of paratyphoid control have been tried in this laboratory. Sulfa drugs in our hands failed to control *Salmonella* infections in groups of experimentally infected mice (Rose and Slanetz, unpublished report). Antibiotics such as penicillin and streptomycin so far have proved very limited in their usefulness as agents to eliminate infection but appear in some instances to prevent the spread of *Salmonella enteritidis* among colonies of mice (Slanetz, 1946). *Salmonella typhimurium* apparently is more resistant to antibiotics than *S. enteritidis*. The use both of sulfa drugs and antibiotics is highly objectionable in any mouse colony from which mice are supplied for experimental purposes, since experimental results may be adversely influenced by their administration.

In this laboratory, observations on the control of *Salmonella* infection among laboratory animals have been in progress for over 17 years. These have been chiefly concerned with the reliability of fecal cultures compared to other methods used in the detection of *Salmonella* carriers.

Buchbinder *et al.* (1935) published observations on enzootic paratyphoid infection in a rat colony. This work yielded a method for the control and elimination of paratyphoid infection in laboratory colonies of rats. Even though this method of control proved highly successful with the rat, when applied to the mouse it soon became evident that a modification of the procedure would be necessary adequately to control and practically to eliminate *Salmonella* infection in breeding colonies of mice having a high percentage of carriers. This laboratory has been particularly fortunate in being invited to do mouse typhoid control work in connection with the Japanese B encephalitis program during the last war and in acquiring information relative to the incidence of *Salmonella* carriers in 20 commercial mouse colonies supplying animals for the project. The accumulation of data on the results of tests in breeding colonies of mice here at the college and in the field now warrants the statement that a practical method of paratyphoid control has been developed. This method has been adopted as a routine procedure by a commercial mouse breeder as well as by this laboratory.

Fecal cultures from the living animal. A pellet of feces is collected directly from an individual mouse in a test tube containing 1 to 2 ml of 1 per cent peptone water and a sterile swab. When it is not possible to obtain a pellet of feces, the rectum of the mouse is swabbed, the 2 ml of peptone water in the tube discarded, and the wet swab placed in the sterile tube preparatory to streaking on brilliant green agar. Comparative tests on 130 paratyphoid-positive mice indicate that the swab method gives as many positive cultures as the fecal pellet method.

The feces are broken up by the sterile swab into a fine suspension and then streaked on brilliant green agar plates by means of the wet swab. A fairly heavy inoculation is desirable. After overnight incubation at 37 C the plate cultures are examined for the presence of *Salmonella*-like colonies. All suspicious growths and colonies are tested by slide agglutination with a specific antiserum. The composition of the brilliant green agar medium is as follows: 2 per cent extract agar pH 7.4 to 7.6, 1.0 per cent lactose, 0.1 per cent glucose, 1 per cent Andrade's solution, and brilliant green dye in a final dilution of 1 to 262,500. The dye dilution is greater than that used in most brilliant green mediums, but it was found that greater concentrations of brilliant green dye, 1:100,000 and 1:150,000, gave negative results with many known positive mice. The use of enrichment broths failed to increase the number of positive fecal cultures obtained in a specially selected group of 200 mice, 80 per cent of which were continuous excretors of *S. enteritidis*. Various media for the isolation of *Salmonella* were tried but these showed no advantages over the brilliant green agar medium.

To effect economy of time and supplies some laboratories streak composite samples of feces from 2 to 5 mice. Our work indicates that it is necessary to sample each mouse individually, one fecal pellet per sterile tube. When a composite sample from 2 to 5 mice is streaked, overgrowth on the incubated plate frequently obscures a positive culture. Comparative tests on 800 mice of which 4 per cent were *Salmonella* excretors by the single-pellet one-tube method, showed that the composite sampling method yielded fewer positive cultures.

Agglutination tests. The slide agglutination tests were made with a 1:50 dilution of combined *S. enteritidis* and *S. typhimurium* antiserums. The cultures agglutinated by the serum and those giving doubtful reactions were routinely inoculated into Durham fermentation tubes containing glucose, lactose, and sucrose. Films were also made of the cultures and stained by the method of Gram.

Pathogenicity tests. The *Salmonella* cultures isolated from feces and organs were tested for pathogenicity in mice by injecting 0.1 and 0.5 ml of 24-hour nutrient broth culture intraperitoneally.

Organ cultures from dead animals. Pieces of liver and spleen were placed on moist extract agar plates and on brilliant green agar plates. When growth occurred, the culture was tested by the slide agglutination method and fermentation tests. If the plates showed no growth after 1 week's incubation, the material was regarded as negative.

RESULTS

Selection of suitable breeding stock for testing. The use of the feces culture method has enabled us to arrive at a satisfactory method of *Salmonella* infection control in breeding colonies of mice and with the practical elimination of infected

TABLE 1

Results of fecal cultures on samples taken routinely at weekly intervals

DATE	NO. OF SAMPLES	NO. PARATYPHOID-POSITIVE
October, 1947	638	4
November	570	5
	450	0
	400	0
December	500	0
	580	4
	500	2
January, 1948	359	1
	360	4
	360	6
February	490	1
	360	1
	360	0
	360	0
March	360	0
	360	1
	360	8
	360	0
	360	0
April	360	0
	720	0
	720	1
	720	3
May	720	0
	720	1
	720	3
	450	2
June	450	2
	450	2
	450	1
	450	0
	400	0
July	450	0
	450	0
	450	0
August	450	0

stock. Over a period of 17 years more than 120,000 individual tests on mice have been made to determine the most effective procedures. During the past 5 years two procedures have been tried on a large scale with encouraging results. One method consisted of making fecal cultures on individual weanling mice,

weighing about 12 g, to be used as breeders. The level of infection under this system remained at about 2 per cent for a period of several years. Large-scale testing was next done on young adult mice, females at about 20 g in weight and males 21 g, approximately one week before mating. This procedure proved to be much more effective than that involving weanling mice. The carrier incidence on one series of 16,000 young adult mice was under 0.4 per cent.

Tests on stock showing high percentage of carriers. Our results on tests of mice in colonies in which 10 per cent or more of the animals are *Salmonella* carriers show that the single test method must be modified. Initially in such cases three successive tests on the same mouse within a period of 10 days are indicated. After the initial testing is done, monthly tests on all breeders should be performed until several consecutive tests are negative.

Comparative tests on weanlings and young adults indicate that the older mouse is more frequently a carrier. This is in agreement with the findings of

TABLE 2

Comparative results with fecal and organ cultures in the detection of Salmonella infection in mice

	DURING LIFE		POST MORTEM	
	No.	*%*	*No.*	*%*
Total number of animals......	50		50	
Feces positive	50	100	50	100
Liver positive..			27	54
Spleen positive			24	48
Agglutinins* present and feces positive.......	9	18		
Agglutinins* present and spleen positive.....			8	16

* Serum dilution 1:40.

Buchbinder *et al.* (1935) on paratyphoid infection in young and old rats. We have found that some weanling mice from mothers that are continuous excretors of *Salmonella* organisms often give negative fecal tests, but at the age of 3 to 4 months many of these same mice become infected and excrete *Salmonella* organisms. Obviously, for the practical control of infection, mice of breeding age should be tested just before they are placed in mating cages.

In table 1 are summarized the findings obtained on such mice in routine weekly tests of a commercial mouse colony which has been under our bacteriological supervision for a period of 10 years. The carrier incidence, 0.35 per cent, is exceptionally low. No seasonal influence is evident from the results obtained by the feces culture method. All positive mice were retested and any 10-mouse unit cage having a positive mouse was removed from the colony and the animals were destroyed. It is of interest that a random sampling of 500 young adults in this commercial mouse colony yielded only 1 positive fecal culture. A further check on the incidence of paratyphoid infection in this colony was made by culturing livers and spleens of 200 mice. Negative results were obtained on these mice.

In table 2 a summary is given on the comparative results of cultures and agglutinations on a group of mice which were excretors of *Salmonella* organisms. Relatively few of the mice had agglutinins in a serum dilution of 1:40 and not all showed positive organ cultures.

SUMMARY

A practical method for the control of *Salmonella* infection in breeding colonies of mice has been developed in the feces culture method.

An incidence of less than 0.4 per cent of paratyphoid carriers was obtained in a large commercial mouse colony by the individual-mouse single-test method.

To maintain a low incidence of *Salmonella* infection in a breeding colony it is necessary to test young adult mice weighing approximately 20 grams prior to mating. Use of weanling mice resulted in a higher percentage of carriers.

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TRACER STUDIES ON THE ROLE OF ACETIC ACID AND CARBON DIOXIDE IN THE FERMENTATION OF LACTATE BY CLOSTRIDIUM LACTO-ACETOPHILUM

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In a previous study of the fermentation of lactate by *Clostridium lacto-acetophilum* (Bhat and Barker, 1947) it was shown that pure cultures of this organism can decompose lactate only when acetate is provided as a second substrate, whereas enrichment cultures of the same organism do not require added acetate. This difference in nutritional requirements indicated a corresponding difference in the catabolic processes occurring in the two types of cultures. In pure cultures, lactate and acetate disappeared while butyric acid, carbon dioxide, and hydrogen were formed, whereas in enrichment cultures lactate was decomposed with the formation of acetate in addition to the other products. It is also significant that the yield of carbon dioxide was much lower in the enrichment cultures. The reduced yield of carbon dioxide taken in conjunction with the formation of acetate indicated that the organisms in the enrichment cultures were using carbon dioxide as an oxidant and were converting it to acetic acid. In pure cultures the high yield of carbon dioxide and the requirement for acetate indicated that the bacteria were unable to reduce carbon dioxide in this way.

The tracer experiments described in the present paper were designed to provide a direct test of the conclusions derived from the above-mentioned nutritional and metabolic experiments.

EXPERIMENTAL RESULTS

In the first experiment (table 1, experiment 1) lactate was fermented by an enrichment culture in the presence of C^{14}-labeled bicarbonate. At the end of the fermentation, the isotope was found in acetate, butyrate, and the residual bicarbonate. This proves that the bacteria converted carbon dioxide to acetate.[2] The data also indicate that this was a quantitatively important reaction in the fermentation. The labeled butyrate was undoubtedly formed from labeled acetate, as is demonstrated in experiment 3 (table 1).

In the second experiment (table 1, experiment 2) lactate was fermented by a pure culture of *C. lacto-acetophilum* in the presence of labeled bicarbonate. Only a relatively insignificant amount of the labeled carbon was found in the

[2] The distribution of C^{14} in the fatty acids was not determined, but in view of the results obtained with other bacteria (Barker, Kamen, and Haas, 1945) it is probable that all the carbon atoms were labeled.

volatile acids. This confirms the previous conclusion that pure cultures cannot use carbon dioxide as an oxidant.

The third experiment (table 1, experiment 3) consisted of a pure culture fermentation of lactate in the presence of labeled acetate. The butyrate formed in the fermentation was radioactive, showing that it had been formed from acetate. Furthermore, the specific activity of the acetate was greatly reduced during the fermentation. This proves that acetate was formed from lactate even though there was a net disappearance of acetate. From the magnitude of the change in specific activity it was calculated by the method previously described (Barker, Kamen, and Haas, 1945) that approximately one mole (actually 0.91 mole) of acetate was formed per mole of lactate decomposed. This is consistent with the view that all the lactate undergoes an oxidative decarboxylation. The slight radioactivity in the final bicarbonate shows that little or no oxidation of acetate occurred.

TABLE 1

Carbon dioxide and acetate utilization by pure and enrichment cultures of C. lacto-acetophilum

	EXPERIMENT 1 ENRICHMENT CULTURE		EXPERIMENT 2 PURE CULTURE		EXPERIMENT 3 PURE CULTURE	
	mM/100 ml	cts/min/mM	mM/100 ml	cts/min/mM	mM/100 ml	cts/min/mM
Substrates						
Lactate decomposed . ..	8.50		3.74		6.77	
Acetate, initial..			6.20		4.38	5,100
Bicarbonate, initial .	1.40	10,600	0.53	10,700		
Products						
Acetate, final	3.90	1,350	2.52	} 65 {	1.76	1,450
Butyrate, final.....	4.29	1,650	3.28		4.59	4,250
Bicarbonate, final...... ..	2.62	570	3.97	1,220	6.47	40
Hydrogen formed .	12.5		2.48		4.83	

The experimental results demonstrate that there is a striking difference in the chemical reactions occurring in enrichment and pure cultures of *C. lacto-acetophilum;* the former utilize carbon dioxide for the synthesis of acetic acid whereas the latter do not. So far we do not know the reason for this difference. The most obvious explanation is that the enrichment cultures contain one or more organisms that are able to utilize carbon dioxide. As yet, however, all attempts to isolate bacteria from the enrichment cultures which either alone or in combination with *C. lacto-acetophilum* can reduce carbon dioxide and, consequently, ferment lactate in the absence of added acetate have been unsuccessful. *Butyribacterium rettgeri* can cause a fermentation of this type (Barker, Kamen, and Haas, 1945), but it has never been found in these cultures.

EXPERIMENTAL PROCEDURES

The experiments were done with growing cultures. In the pure culture experiments, *C. lacto-acetophilum*, strain 3, was used (Bhat and Barker, 1947).

In the enrichment culture experiment, the sterilized medium was inoculated with garden soil. All cultures were incubated at 37 C until growth ceased.

The basal medium contained the following compounds in grams per 100 ml of glass-distilled water: sodium lactate, 1.0; yeast autolyzate, 0.3; sodium thioglycolate, 0.05; $(NH_4)_2SO_4$, 0.05; K_2HPO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.002; and $CaSO_4 \cdot 2H_2O$, 0.001; pH 7.0. To this medium was added synthetic carboxyl-labeled acetate or labeled sodium carbonate in the amounts indicated in table 1. The acetate was added before autoclaving, whereas the sodium carbonate was added afterward as a sterile solution. The fermentations were conducted in an all-glass vessel with an outlet for collecting the evolved gases over mercury. Oxygen was excluded by the use of an "oxsorbent" seal.

The fermented media were analyzed by methods previously used in this laboratory (Bornstein and Barker, 1948). Acetic and butyric acids were separated by azeotropic distillation. Radioactivity measurements were made by the technique described by Kamen (1947).

SUMMARY

By the use of C^{14} it has been shown that the bacteria in enrichment cultures of *Clostridium lacto-acetophilum* use carbon dioxide as an oxidant, converting it into acetic and butyric acids. In pure culture this organism is unable to reduce carbon dioxide, but it oxidizes lactate to acetate and carbon dioxide and then converts the acetate to butyrate.

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THE NUTRITIONAL REQUIREMENTS OF CLOSTRIDIUM ACETICUM[1]

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Wieringa (1936; 1939–1940) described the isolation of a soil anaerobe, *Closstridium aceticum*, which was able to convert hydrogen and carbon dioxide to acetic acid. The nutritional requirements of this organism appeared to be complex, an extract of mud being an indispensable constituent of the medium. We have made further investigations of the nutrition of *C. aceticum*, which have led to the identification of the essential nutrients, although some stimulating factors remain to be characterized.

EXPERIMENTAL RESULTS

The experiments were done with a strain of *Clostridium aceticum* obtained from Dr. K. T. Wieringa, to whom the authors are greatly indebted.

The basal medium contained the following compounds in grams per 100 ml of glass-distilled water: K_2HPO_4, 0.2; $(NH_4)SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; $CaSO_4$, 0.0015; $FeSO_4 \cdot 7H_2O$, 0.00025; $MnSO_4 \cdot 4H_2O$, 0.00005; $Na_2MoO_4 \cdot 2H_2O$, 0.00005; sodium thioglycolate, 0.05; and Na_2CO_3, 1.0. The pH was adjusted to 7.8 with hydrochloric acid after the medium was autoclaved. Anaerobic conditions were maintained by the use of a potassium-carbonate-pyrogallol seal.

When 0.3 per cent Difco malt extract and 0.2 per cent Difco yeast extract were added to the basal medium, satisfactory growth was generally obtained.[3] The malt extract could be replaced by glucose without much reduction in growth, and casein hydrolyzate plus a mixture of ten growth factors of the B complex could be satisfactorily substituted for the yeast extract. By successive elimination the required growth factors were identified as biotin, pyridoxamine, and pantothenic acid. Pyridoxine was less potent than pyridoxamine. Finally, glutamic acid was substituted for the casein hydrolyzate, but this resulted in slower growth and reduced final yield of cells. However, more satisfactory growth was obtained on subsequent transfers in this medium.

Table 1 shows the relative growth in media of different composition as determined with the Evelyn colorimeter. The growth factors referred to in the table

[3] The growth of the organism is sometimes erratic, particularly when small inocula are used. At times there is a long delay before growth starts in media which on other occasions are entirely satisfactory. This is true for both complex and simplified media of the type described in this paper. It is therefore believed that this erratic behavior does not affect the validity of the conclusions we have drawn from the experiments described.

were used in the following concentrations: thiamine hydrochloride, riboflavin, and calcium pantothenate, 2 µg per ml; nicotinic acid, nicotinamide, pyridoxine hydrochloride, and pyridoxamine dihydrochloride, 1 µg per ml; *para*-aminobenzoic acid, biotin, and folic acid, 0.01 µg per ml. Cultures with yeast extract reached maximum growth in 4 days, with casein hydrolyzate in 7 days, and with glutamic acid in 9 days.

In order to prove that we were dealing with *C. aceticum* throughout these experiments and that the results were not complicated by the presence of a contaminant, single colony isolations were made from the final glutamic acid medium. The colonies were tested for purity and the organisms were shown to

TABLE 1

Growth of Clostridium aceticum in a medium containing 1 per cent glucose and other nutrients

MEDIUM	(2—log G)· 100 MAX. TURBIDITY
0.3% M. E. + 0.2% Y. E....	14
0.2% Y. E................	8
0.1% C. H. + 10 vitamins........	11
0.1% C. H. + biotin + pyridoxamine + pantothenate........	12
0.1% C. H. + pyridoxamine + pantothenate..........	0
0.1% C. H. + biotin + pantothenate....................	0
0.1% C. H. + biotin + pyridoxamine....	2
Biotin + pyridoxamine + pantothenate......	0
0.04% glutamate + biotin + pyridoxamine + pantothenate....	5

M. E. = Difco malt extract; Y. E. = Difco yeast extract; C. H. = "Vitamin-free" acid-hydrolyzed casein (Nutritional Biochem. Corp.).

possess the same morphological and growth characteristics as the original culture. They also were shown to produce acetate from bicarbonate and gaseous hydrogen when a yeast-extract malt-extract medium was used.

SUMMARY

Clostridium aceticum has been shown to grow in a synthetic medium containing glucose, glutamic acid, biotin, pyridoxamine, and pantothenic acid. Some stimulatory factors are still unidentified.

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MICROORGANISMS IN THE CECAL CONTENTS OF RATS FED VARIOUS CARBOHYDRATES AND FATS[1,2]

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The nutritional significance of intestinal microorganisms was indicated by early work (Cooper, 1914; Theiler *et al.*, 1915), and during recent years it has been established that all the vitamins of the B complex, as well as vitamin K, may be synthesized in the alimentary tract of animals as a result of the activity of these intestinal organisms (Wegner *et al.*, 1941; Day *et al.*, 1943; Elvehjem, 1946). In addition, information has been obtained concerning the effect of different carbohydrates on the intestinal synthesis of B vitamins (Elvehjem and Krehl, 1947). It is fairly well known now that irrespective of what B vitamin is investigated its requirement is reduced when relatively insoluble carbohydrates like dextrin or starch are used in the diet. Lactose sometimes favors the production of some of the B vitamins, but glucose, sucrose, or other readily assimilable sugars are without any effect in this respect (Fridericia *et al.*, 1927; Guerrant *et al.*, 1934a,b, 1935, 1937; Morgan *et al.*, 1938; Schweigert *et al.*, 1945; Luckey *et al.*, 1946; Sarma *et al.*, 1946; Teply *et al.*, 1947; Couch *et al.*, 1948). The effect of different carbohydrates in encouraging or suppressing a vitamin-synthesizing intestinal flora is not unexpected in view of the fact that a carbohydrate-rich diet stimulates an aciduric flora, whereas meat or a high protein diet favors a proteolytic flora and large numbers of coliform organisms.

As the cecum has been postulated to be the main site of vitamin synthesis by intestinal microorganisms (Moro, 1905; Tissier, 1908; Torrey, 1919; Cannon *et al.*, 1920; Porter and Rettger, 1940; Taylor *et al.*, 1942; Schweigert *et al.*, 1945), all microbiological work in the present study was done on cecal contents. Of the various carbohydrates studied, lactose and dextrin have been found to have the most marked effect in favoring an aciduric flora (Cannon, 1920; Porter and Rettger, 1940). Interesting differences in the types of different intestinal organisms of animals on various carbohydrates have also been found recently (Johansson *et al.*, 1947; Gall *et al.*, 1948a,b).

The effects of the fat component of the diet on the intestinal flora of animals have received little attention, but, as pointed out by Elvehjem and Krehl (1947), recent investigations clearly indicate the role of fat in modifying the requirement for some of the B vitamins, especially thiamine, riboflavin, niacin, biotin, pantothenic acid, and pyridoxine.

The present investigation was undertaken to determine the effects of carbo-
hydrates and fats upon the cecal flora of the rat. This work was done in an
attempt to develop a satisfactory explanation for the different growth-promoting
activities of diets containing (a) different carbohydrates and fats and (b) differ-
ent levels of fats.

EXPERIMENTAL METHODS

All experiments were performed with a "parasite-free" strain of rats obtained
from Sprague Dawley Company, Madison, Wisconsin; the rats were individually
caged in the new type of coprophagy-preventing "tube cage" first developed by
Geyer *et al.* (1947). This was done in an attempt to obliterate variations in the
vitamin intake of the animals. The diet consisted of alcohol-extracted casein,
20 per cent; salt mixture IV, 4 per cent; fat (butterfat or corn oil), 10 per cent or
28 per cent; and carbohydrate (sucrose, lactose, or dextrin), 66 per cent with
low fat diet or 48 per cent with high fat diet. The fat-soluble vitamins were
incorporated into the fat, whereas the water-soluble vitamins were added to
the main batch of basal ration during the mixing of casein, salt mixture, and
carbohydrate. The normal level of vitamin supplement per 100-g ration was
as follows:

Thiamine.............................	0.20 mg
Riboflavin..........	0.30 mg
Pyridoxine.................	0.30 mg
Ca-pantothenate...........................	1.50 mg
Choline hydrochloride...	150.00 mg
Beta-carotene (90 per cent beta-, 10 per cent alpha-carotene)	0.56 mg
Alpha-tocopherol......	2.24 mg
Calciferol (crystalline, irradiated ergosterol).............	0.014 mg
2-Methyl-1,4-naphthoquinone........................	0.21 mg

The experiments were conducted for different periods varying from 2 to 6
weeks, after which the animals were killed and analyzed.

Samples of cecal contents for analysis were obtained by opening the abdomen
of the animal under aseptic conditions, removing the cecum to a sterile petri
dish, and squeezing out the total cecal contents with sterile, smooth forceps by a
"milking process." In most cases a record was kept of the total cecal contents of
the animal. After proper mixing, 0.5 g of the cecal contents were transferred
to a sterile 50-ml water blank containing glass beads. After vigorous and
thorough shaking of this original suspension (1×10^{-2}), it was further diluted
to 10^{-4} and then serially by steps of 100 until a dilution of 10^{-8} was obtained.
Quantitative inoculation was then made of the respective dilutions into various
culture media.

An estimate of the total viable population of aerobic and anaerobic bacteria
was made by using the BBL thioglycolate medium. In a few cases, the total
aerobic plate count was determined by the use of a tryptone glucose yeast ex-
tract agar medium. For culturing anaerobes, the oat jar method was used, and a
period of 48 to 60 hours at 37 C was allowed for incubation of the plates. Inocula

used in both aerobic and anaerobic plate counts ranged from 10^{-5} to 10^{-8} in duplicate.

Difco EMB agar was used as the plating medium for culturing coliform organisms, and the dilutions of inocula ranged from 10^{-3} to 10^{-7}. An incubation period of 48 to 60 hours at 37 C was allowed for the growth of these organisms.

The "SF" medium of Hajna and Perry (1943), which utilizes 0.5 g per liter of Na_3N, as well as a selective incubation temperature of 45 C for the inhibition of organisms other than enterococci (and other intestinal streptococci which grow at 45 C), was used for enumerating this group. The tube dilution count method was adopted in this case; five tubes were inoculated per dilution and the counts calculated by the most probable number method.

Shake tubes of carrot liver extract medium introduced by Foster et al. (1942) were used for counting "lactics," and the dilutions of inoculum ranged from 10^{-5} to 10^{-9}. This medium was found to be selective for this group in view of the fact that at such high dilutions no other organisms were found to be present. It was possible to base these counts on numbers of colonies developing in the tubes.

RESULTS AND DISCUSSION

Relation of carbohydrates and fats of the diet to the cecal flora of rats. Results of the analyses of the cecal flora of rats on diets containing one of the three carbohydrates, sucrose, lactose, or dextrin, and either of the fats, butterfat or corn oil, are presented in tables 1 to 3. It is apparent from these data that a considerable amount of variation exists in the size and the composition of the microbial population of the cecum in the different groups of animals, even when they are maintained under almost identical dietary and other environmental conditions. In no case was it possible to obtain an exact duplication of the results obtained with any group of rats on a particular diet. The same extent of variation is also observed in the individual animals belonging to a group kept under the same dietary and environmental conditions, and therefore in order to come to any logical conclusions one must consider large numbers of animals in each group. The results given in the tables are based in most cases on analyses of the cecal contents of a series of 4 to 6 rats in each group. However, it has been observed that, even when the numbers of the organisms in the ceca of different animals belonging to the same group varied to a great extent, the relative proportions of the different kinds of organisms in the cecum of each animal were approximately the same.

The effects of the various carbohydrates on the microbial flora of the cecum is apparent in these results. In all cases the lactic group of organisms dominated the other organisms; this result seems to support the reports by most other workers in this field. However, the ratio of this group of organisms to some other group like coliforms depended to a great extent on the carbohydrate of the diet.

In the study on the influence of various types of carbohydrates, much higher aerobic and anaerobic plate counts were found in the cecal contents of rats on

the lactose diet as compared to that of rats on sucrose or dextrin diets.   Of the 10 groups of animals on the lactose diet, in only 4 cases did the total count of cecal contents fall below a billion per gram, whereas on a sucrose diet only 1 out of the 12 groups studied yielded total counts above 1 billion per gram.   On the dextrin diet, the average total count of cecal contents of all the animals was much below 1 billion per gram.   Moreover, when the comparison is made on the basis of total number per cecum, the result is even more striking.   A glance at the column giving the weights of the total cecal contents reveals that the lactose-fed animals had more total cecal contents: on the average, two to three times more cecal contents were found in lactose-fed animals than in those fed a sucrose

TABLE 1

Cecal flora of rats on diets containing sucrose as the dietary carbohydrate

(Counts indicate number of organisms per gram of fresh cecal contents)

TIME OF EXPERI-MENT (WEEKS)	VITAMIN LEVEL*	IN-CREASE IN WT (G)	NO. OF RATS	AVG WT OF THE CECUM (G)	AEROBIC PLATE COUNT	ANAEROBIC PLATE COUNT	COLIFORMS	LACTICS
Group I.   Fat—*Butterfat*
2	A	41	5	0.632	215,000,000	398,000,000	220,000	199,800,000
4	A	84	5	0.889	60,000,000	248,600,000	222,000	95,700,800
6	A	122	5	1.095	194,000,000	221,900,000	528,000	181,800,000
6	A	119	5	—	107,340,000†	—	690,000	130,000,000
6	B	150	5	—	6,146,000,000†	—	1,040,000	268,000,000
9	A	168	5	—	75,875,000	62,575,000	391,000	170,250,000
Group II.   Fat—*Corn oil*
2	A	452	5	0.487	260,700,000	473,800,000	1,030,000	183,200,000
4	A	709	5	0.785	161,125,000	335,000,000	164,250	229,500,000
6	A	106	4	0.996	342,750,000	456,250,000	383,000	1,173,000,000
6	A	110	5	—	92,730,000†	—	496,000	152,500,000
6	B	128	5	—	93,100,000†	—	1,572,000	245,800.000
9	A	149	4	—	157,000,000	224,250,000	570,000	432,000,000

* Vitamin level A is normal; vitamin level B is approximately twice normal.
† Tryptone glucose yeast extract agar medium was used for counting.

or dextrin diet.   Thus the total numbers of organisms per cecum was higher in rats fed lactose than in those fed sucrose or dextrin.

There was also a significant difference in the aerobic to anaerobic plate count ratio in the flora caused by feeding the three carbohydrates.   It was nearly 1:1 on a dextrin diet, less than 1:2 on the sucrose diet, and less than 1:4 on the lactose diets.

Not only did the total aerobic and anaerobic plate counts run high on the cecal contents of rats on a lactose diet, the numbers of coliforms and lactics were also very high.   The effect of lactose in inducing a high aciduric flora is known from very early times, but its effect in maintaining a high coliform flora has not been reported by most of the early workers.   The chief effect of a lactose

TABLE 2

Cecal flora of rats on diets containing lactose as the dietary carbohydrate

(Counts indicate number of organisms per gram of fresh cecal contents)

TIME OF EXPERIMENT (WEEKS)	VITAMIN LEVEL*	INCREASE IN WT (G)	NO. OF RATS	AVG WT OF THE CECUM (G)	AEROBIC PLATE COUNT	ANAEROBIC PLATE COUNT	COLIFORMS	LACTICS
				Group I.	Fat—*Butterfat*			
4	A	21	5	3.37	4,150,000,000	8,220,000,000	1,523,000,000	8,520,000,000
6	A	88	5	3.88	1,248,000,000	6,334,000,000	396,000,000	29,500,000,000
6	A	86	5	—	1,680,000,000†	—	270,000,000	12,530,000,000
6	A + biotin + folic acid	103	5	—	322,000,000†	—	192,683,500	4,550,000,000
6	B	98	5	—	870,000,000†	—	170,960,000	22,330,000,000
				Group II.	Fat—*Corn oil*			
4	A	14	5	3.07	5,910,000,000	11,510,000,000	1,800,000,000	22,380,000,000
6	A	72	5	3.27	2,418,000,000	10,870,000,000	130,650,000	12,340,000,000
6	A	80	5	—	358,900,000†	—	85,670,000	1,026,000,000
6	A + biotin + folic acid	81	4	—	1,588,000,000	—	367,000,000	2,830,000,000
6	B	82	4	—	123,000,000†	—	4,272,000	1,822,000,000

* Vitamin level A is normal; vitamin level B is approximately twice normal.
† Tryptone glucose yeast extract agar medium was used for counting.

TABLE 3

Cecal flora of rats on diets containing dextrin as the dietary carbohydrate

(Counts indicate numbers of organisms per gram of fresh cecal contents)

TIME OF EXPERIMENT WEEKS)	VITAMIN LEVEL*	INCREASE IN WT (G)	NO. OF RATS	AVG WT OF THE CECUM (G)	AEROBIC PLATE COUNT	ANAEROBIC PLATE COUNT	COLIFORMS	LACTICS
				Group I.	Fat—*Butterfat*			
2	A	44	4	0.865	233,000,000	274,000,000	40,620,000	440,000,000
4	A	89	3	1.32	175,600,000	117,000,000	1,598,300	132,830,000
6	A	133	5	2.00	557,500,000	588,400,000	12,365,000	650,000,000
				Group II.	Fat—*Corn oil*			
2	A	43	4	0.91	215,250,000	222,700,000	7,705,000	459,250,000
4	A	86	4	1.35	183,400,000	195,000,000	5,672,000	180,000,000
6	A	132	4	1.48	177,000,000	182,500,000	7,230,000	250,000,000

* Vitamin level A is normal.

diet, and in general of a diet containing any carbohydrate, has been reported to be a suppression of the proteolytic organisms, including some coliforms, but in view of the fact that the latter are lactose fermenters, there is no reason why they should be suppressed. Kendall (1911–1912) and Cruikshank (1928), as a result of their *in vitro* experiments, suggested long ago that feeding a large amount of a lactose diet might first lead to an abundant growth of *Escherichia coli*, *Clostridium perfringens*, and other carbohydrate fermenters, and that the acid developed might offer a suitable medium for the growth of lactobacilli. However, this theory was strongly opposed by Rettger and Cheplin, who observed almost complete elimination of *E. coli* and *C. perfringens* under these conditions.

Dextrin also seems to encourage a large coliform flora, though its effect appears to be much less than that of lactose. The influence of dextrin in maintaining a high coliform count in chicks has also been reported by Evenson (1947) and by Johansson, Shapiro, and Sarles (1947). In contrast to the reports by many workers, the effect of dextrin in maintaining a high aciduric flora could not be demonstrated.

The counts of all types of organisms studied were very low in the ceca of animals fed sucrose compared to those of animals fed on the other two carbohydrates.

When a comparison is made on the basis of organisms present per gram of the cecal contents, no significant difference in the numbers of different kinds of bacteria appears to be present when the groups of animals receiving either butterfat or corn oil are compared, except for the fact that the enterococcus group was somewhat lower in animals on the butterfat diet. The values of the numbers of these organisms are not included in the tables as not enough experiments were performed to warrant definite conclusions. It was, however, observed that butterfat-fed rats, in almost all cases, had heavier ceca, and hence when a comparison is made on the basis of the number of organisms present per cecum, the butterfat groups had higher numbers of each kind of cecal organisms than those of the corn oil groups.

In view of the somewhat higher amounts of oleic acid in corn oil, which has been reported to have a growth-stimulating effect on *Lactobacillus casei* (Williams and Fieger, 1946), one might expect a higher number of lactic organisms in the cecal flora of rats receiving corn oil. No such effect was observed in any of the groups on corn oil with dextrin or lactose as the dietary carbohydrate, but on the sucrose diet there seemed to be slightly higher numbers of lactic organisms in corn-oil-fed animals. It is probable that on dextrin and lactose diets the effect of the oleic acid of corn oil, if there is any, is masked by the much stronger influence of the carbohydrates themselves. Reports by Evenson (1947) that the coliforms are suppressed by high butterfat diets could not be confirmed in the present study.

A tentative explanation of the difference in growth-promoting values of the three carbohydrates can be offered by considering the different types of organisms encouraged by them. However, it should be mentioned that the basic idea of such an interpretation lies in the correlation of the physiological behavior

of the different carbohydrates and fats (as measured by the growth of the animal) with the changes in numbers of the most important groups of organisms, some of which are considered to be vitamin synthesizers, whereas certain others are utilizers of the vitamins available to the animal. From the standpoint of vitamin economy of the animal host, the three most important groups of intestinal bacteria are the coliforms, anaerobes, and lactics. Many investigators are of the opinion that intestinal synthesis of the vitamins is carried on by the coliform organisms to the greatest extent, and so this group deserves to receive the most careful attention from the nutritionist's point of view. Anaerobic bacteria have been reported by Benesch (1945) to utilize some of the vitamins already synthesized by the coliforms; hence this group as well as the lactic group of organisms, which require almost all the known growth factors for their growth and multiplication, may be regarded as agents harmful to the animal host. Though Metchnikoff has described the lactobacillus group of bacteria as the promoter of intestinal hygiene, Mitchell (1927) has expressed the contrary opinion, in view of the finding that the rate of growth of rats on different carbohydrates varied inversely with the percentage of the aciduric group of bacteria in the cecum. She concluded that an aciduric type of intestinal flora in itself does not ensure a good general condition of the animal over a long period of time if a high percentage of lactose or other slowly absorbed carbohydrate has been used to support the aciduric flora. If the aciduric flora can be supported by smaller amounts of lactose, the growth of the animal might then remain normal.

It is not out of place to point out here that the main defect in the approach to the problem employed in our work lies in the fact that, through such a study, no information can be obtained on which vitamins are actually synthesized and in what quantities. In addition, it is not possible to determine the kinds or quantities of vitamins liberated in the cecum or finally absorbed by the host animal. It is also definite that all the different organisms of the coliform group are not equally efficient vitamin synthesizers. Furthermore, it is not fair to assume that all the lactics and anaerobes are harmful agents that utilize or destroy the vitamins synthesized by the coliforms. It is also possible that there might be other, unidentified bacteria which, though present in smaller numbers, might exert a strong influence on the nutrition of the animal. Thus a complete picture of what is going on in the intestinal tract of the animal can only be secured when the information obtained by a quantitative study of the different organisms is supplemented by cultural studies of the predominant organisms belonging to each group, followed by testing of their vitamin-synthesizing capacities.

The nearly identical influence of the dietary fats, butterfat and corn oil, on the growth of young rats on a sucrose diet was reflected in the composition of their cecal floras, which were found to be practically identical. On a dextrin diet, too, there was hardly any difference in the cecal flora when the dietary fat consisted of either butterfat or corn oil. Whether the slightly higher numbers of anaerobic organisms found in the ceca of lactose-corn-oil-fed animals are respon-

sible for the inferiority of this diet as compared to that containing butterfat, or whether some biochemical abnormalities associated with a lactose diet are counteracted to a greater extent by butterfat than by corn oil, can only be settled through future research.

Effects of the level of fat of a sucrose-containing diet on some of the cecal microorganisms. Comparatively few kinds of bacteria have the ability to split fat, and probably small numbers of those normally present in the intestinal tract possess this property. Therefore the addition of more fat to the diet is not expected to induce increases in numbers of intestinal bacteria, but on the contrary tends to suppress some of those already present. That this is what happens to the microorganisms of the cecum upon the addition of more fat in a sucrose diet is clearly seen from the results in table 4, which indicate that there is a decrease in the total numbers of organisms when the fat intake of the animal is

TABLE 4

Cecal flora of rats on sucrose diets containing different levels of corn oil

(All counts represent numbers per gram of fresh cecal contents)

EX-PERI-MENT NO.	LEVEL OF CORN OIL (%)	NO. OF RATS	AVG INCREASE IN GROWTH (G)	AEROBIC PLATE COUNT	ANAEROBIC PLATE COUNT	LACTICS	COLIFORMS
I	10	5	169	808,000,000	2,091,000.000	1,230,000,000	12,660,000
	28	5	156	420,000,000	1,474,500,000	872,000,000	926,000
II	10	5	188	873,500,000	2,238,250,000	1,660,000,000	99,323,000
	28	5	169	690,100,000	1,787,700,000	1,942,000,000	7,975,800
	28+ "reticulogen"	5	202	1,416,000,000	3,070,000,000	1,918,000,000	51,890,000
	35	5	140	592,000,000	1,265,200,000	1,320,000,000	46,998,000
III	10	5		3,110,000,000	—	3,840,000,000	11,700.000
	35	5		1,996,000,000	—	2,460,000,000	8,725,000

increased. The anaerobic plate count is also decreased in the same order. In view of the fact that analysis was made of only a few groups of organisms like coliforms and lactics, the results do not show conclusively which types of organisms were most affected. However, there is definite indication that the numbers of coliform organisms are decreased to a considerable extent by increased levels of fat in the diet. There is very little change in the numbers of the lactic group of organisms with increases in fat. The slight decrease observed is probably not statistically significant.

The results of this experiment confirm the report by Torrey (1919), who worked on dogs and observed that the numbers of *E. coli* were frequently reduced, that the streptococcus group was markedly reduced, but that the *Lactobacillus acidophilus* type was the least affected and often increased in relative numbers when the animals were fed a high fat diet. However, according to Mitchell

and Isbell (1942), contrary results were obtained in 1931 by Forti, who observed a decrease in the aciduric flora with an increase of fat in the diet.

Analysis of the group of rats on the 28 per cent corn oil sucrose diet supplemented with "reticulogen" (Lilly liver extract) at the rate of 0.1 unit per day, showed a significant increase in the aerobic plate count and in numbers of the coliform group of organisms, but not of lactics. It should be mentioned here that we gave a supplement of "reticulogen" to this group of rats in an attempt to remove the dietary imbalance, if there was any, resulting from the effects of high amounts of fat. We actually observed an increase in growth of the rats of this group by 33 grams over that of the control group receiving no supplement of "reticulogen." This strongly supports the view that diminished growth on a high corn oil diet is due to inhibition of the intestinal synthesis brought about by microorganisms, and that the action of "reticulogen" might be due to a stimulation of intestinal synthesis.

SUMMARY

For animals on a lactose diet the total aerobic and anaerobic plate counts, as well as the numbers of coliforms and lactics, were much higher than the corresponding counts or numbers of the same organisms in the cecal contents of animals on diets containing either dextrin or sucrose.

Compared to sucrose, dextrin was found to favor higher numbers of coliforms in the cecum.

The ratio of the aerobic to the anaerobic plate count was highest on a dextrin diet and lowest on a lactose diet.

The aerobic and the anaerobic plate counts, as well as the numbers of coliforms, were found to be decreased in the ceca of most of the rats fed sucrose diets containing a high level of corn oil.

Supplementation of a high corn oil sucrose diet with "reticulogen" tends to counteract the inhibitory action of corn oil upon the growth of certain cecal microorganisms.

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AN ANALYSIS OF THE ACTION OF PROFLAVINE ON BACTERIOPHAGE GROWTH[1]

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The bacterium-bacteriophage system offers many advantages for the study of the general principles of the host-virus relationship. The availability of precise quantitative methods for analyzing phage growth makes this system suitable for the study of the nature of the inhibition of virus growth by drugs.

A majority of the compounds with suppressive activity on phage multiplication have likewise shown suppressive effects on bacterial multiplication (Delbrück and Luria, 1943; Spizizen, 1943; Bailey, 1943; Jones and Schatz, 1946; Cohen and Anderson, 1946; Cohen and Fowler, 1947). In these cases the inhibition appeared to be indirect through inhibition of the metabolism of the host, and possibly aspecific. Recently a reverse phenomenon has been reported, namely, increased yields of phage occurring at bacteriostatic concentrations of penicillin (Price, 1947). Temperatures above optimum for the growth of the host inhibit phage growth with some degree of specificity (Luria, 1943). What appears to be a specific inhibitory effect of a number of acridines and an anti-acridine action of ribose nucleic acid has been reported by Fitzgerald and collaborators (1946). The present paper is concerned with the results of further studies on the action of the acridine compound proflavine and with their interpretation in relation to the problem of phage multiplication.

MATERIAL AND METHODS

The bacteriophages studied were members of the "T" series whose common host is *Escherichia coli*, strain B (Delbrück, 1946). Plaque counts by the agar layer method with nutrient agar were used to determine phage activity. The "one-step growth experiment," described in detail by Delbrück and Luria (1942), was the basic technique adopted. In this type of experiment, known numbers of phage particles are mixed with an excess of sensitive bacteria, so that most bacteria adsorb a single particle. After a few minutes the mixture is highly diluted to stop further adsorption. The plaque count at this time, before lysis begins, comprises two sources of "infective centers": the infected bacteria and the free, unadsorbed phage. By determining the number of plaques obtained by plating the supernatant of a centrifuged sample, the number of free particles can be determined and the number of infected bacteria estimated by difference.

The final plaque count, after lysis of all infected cells, is a measure of the total yield of phage. From it the average phage yield per infected bacterium can be calculated.

To determine the actual yields of phage from individual cells, the "single burst experiment" (Burnet, 1929; Delbrück, 1945) was used. Before lysis begins, the infected bacteria are highly diluted and distributed to a large number of small tubes, so that each tube receives on the average less than one infected bacterium. By plating the entire contents of each tube after lysis has taken place, the phage yield from individual bursts is obtained.

To study macroscopic lysis, a "multiplicity of infection" of about four particles per bacterium was found suitable. The "r" mutants of the T-even phages were employed to avoid "lysis inhibition" (Doermann, 1948).

The medium used was M-9[3], in which the generation time of $E. coli$ B is approximately 45 minutes, and the latent periods for lysis by T2, T4, T6, and T7 are 21, 28, 30, and 15 minutes, respectively. In experiments with T4 and T6, 20 μg per ml of L-tryptophan were added to permit phage adsorption (Anderson, 1945).

The proflavine (di-amino-acridinium sulfate, Abbott Laboratories) was dissolved in distilled water and adjusted to pH 7 with sodium hydroxide.

RESULTS

Action of proflavine on free phage and on bacterial growth. Exposure of free phage to proflavine in concentrations sufficient to inhibit phage growth (1.0 mg per 100 ml for T2, 0.35 mg per 100 ml for T6; see below) produced a negligible loss of free phage activity in 60 minutes at 37 C.

Plate counts taken at intervals after the addition of proflavine to growing cultures of $E. coli$ B, similar to those used in one-step growth experiments, showed that in those concentrations in which growth inhibition was present the inhibition was evident 1 hour after the addition of the drug. Complete inhibition of reproduction was produced by a concentration of 0.5 mg per 100 ml (table 1).

Concentration of proflavine preventing production of active bacteriophage. The addition of proflavine to a bacterial culture 2 minutes before the addition of bacteriophage either reduces the average yield of phage or suppresses all phage production, depending on the concentration. The amount of proflavine that, in a one-step growth experiment, completely suppresses the ability of infected bacteria to produce active phage will be called the "completely inhibitory concentration" (C.I. concentration).

The data assembled in table 1 show the C.I. concentrations for T2, T4, T6, and T7 to be 0.35, 0.1, 0.05, and 0.75 mg per 100 ml, respectively. Thus, the least sensitive phage is T7, the most sensitive, T6; T4 and T2 have intermediate sensitivities. Growth of T7 is prevented only by concentrations of proflavine that are completely inhibitory for bacterial multiplication, whereas T6 and

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[3] Formula for M-9 Medium: *Solution A*—KH$_2$PO$_4$ 3 g, MgSO$_4$ 0.2 g, NaCl 0.5 g, NH$_4$Cl 1 g, anhydrous Na$_2$HPO$_4$ 6 g, dissolved in 900 ml of distilled water in the order above. *Solution B*—4 g of glucose dissolved in 100 ml of distilled water. Solutions A and B are sterilized separately and mixed in a ratio of 9:1 as needed. The pH of the final solution is 7.0.

T4 are completely suppressed at concentrations that allow uninhibited multiplication of the host.

To estimate the sensitivity of other phage types we used a screening test consisting of plating on nutrient agar in which increasing concentrations of proflavine were incorporated. In this way gross differences in the effectiveness of proflavine can be demonstrated. T1 and T3 appear to be similar to T7; T5 appears intermediate between T4 and T2.

Each phage type is adsorbed by sensitive bacteria at the same rate in the presence as in the absence of proflavine. T2, T4, and T6, the related large phage

TABLE 1

Growth-limiting concentrations of proflavine

The data for various phages represent the average yield per bacterium in one-step growth experiments, measured at the time of maximum yield. The data for *E. coli* B are percentage ratios between viable counts in the presence of proflavine and viable counts in its absence, the conditions of the measurements being identical with those in the corresponding one-step growth experiments.

PROFLAVINE CONCENTRATION (MG/100 ML)	AVERAGE YIELD IN PERCENTAGE OF YIELD IN CONTROL				GROWTH OF E. COLI B IN 1 HOUR, PERCENTAGE OF CONTROL
	T2	T4	T6	T7	
0.005			91		
0.0125			40		
0.025			8		
0.05		3	*		100
0.1		*	*		100
0.125	55	*			
0.2					50
0.25	18				40
0.3	4				
0.35	*			39	
0.5	*			5	0
0.75				*	

* Decrease of infective centers below input.

types, are all more sensitive to proflavine than the small phages T1, T3, and T7 and differ in absolute sensitivity among themselves. The "r" mutants have the same sensitivities as the corresponding wild types.

It should be noted in table 1 that, as the proflavine concentration is reduced, the average phage yield per bacterium gradually increases. Analysis of individual cell yields of T2 in the presence of 0.25 mg per 100 ml proflavine—a not completely inhibitory concentration—showed that the loss in phage yield could be accounted for by a reduction of the individual yields.

Analysis of the action of completely inhibitory concentrations of proflavine. Phage growth, as studied in the one-step growth experiments, can be divided into

a "latent period" of intracellular reproduction, a "rise period" corresponding to phage liberation, and a "stationary period," after liberation is completed.

If the C.I. concentration of proflavine is added to the growth tube 2 minutes before the addition of phage and the infective centers are assayed at intervals, a progressive fall in the plaque count is found, indicating an actual loss of the adsorbed phage. A number of experiments of this kind were done. Table 2 shows the results of representative experiments in which the plaque counts at various times were determined. Data on the yield in the absence of proflavine are presented in the same table. It should be remembered that plating for plaque count results in diluting the proflavine well below inhibitory concentrations.

The data in table 2 show three characteristics of proflavine action: first, there is no loss of infective centers during the latent period; second, there is a progressive loss of infective centers during the rise period; and third, at the stationary period the plaque count reaches approximately the value of the unadsorbed phage, indicating loss of all adsorbed phage. Moreover, as seen clearly in figure 1, in which the results from several experiments are summarized, the loss of infective centers in the presence of proflavine closely parallels the rise in titer in the control without proflavine. This indicates a parallelism between liberation of phage by lysis in the absence of proflavine and loss of ability to produce active phage in the presence of the drug.

These experiments suggested that in the presence of the C.I. concentration of proflavine the cells may lyse at the same time as they would in the absence of the drug, but release no active phage. This would explain the loss of infective centers and the presence of the unadsorbed phage alone at the end of the rise period.[4]

According to this interpretation, the presence of proflavine should allow lysis to occur after a normal latent period. We tested this point by macroscopic observation of lysis. Parallel cultures with and without proflavine were inoculated with enough phage to give multiple infection (about 4 particles per bacterium). Clearing occurred at the same time in both cultures. Proflavine neither prevents lysis of the host nor causes delay in lysis.

Another type of experiment gave further support to the hypothesis. Bacteria were infected with phage in the presence of the C.I. concentration of proflavine, as in the experiments of table 2. Then, at various intervals, samples were highly diluted with medium containing no proflavine. The concentration of proflavine was thus reduced below the inhibitory level. All samples were incubated and plated at a time when the yield in the control tube had reached the stationary period. If the conclusion previously reached is correct, we may expect a full yield if proflavine is removed during the latent period. With dilution after the lysis of cells has begun, the yield should vary inversely with the time of dilution, since, as the time before dilution is extended, more and more cells will have lysed

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[4] Phage T6 shows the peculiarity that the rate of loss of adsorbed phage in the presence of proflavine is more rapid than the rate of increase in phage titer without proflavine. The latter value, however, can only be considered as an estimate of the number of bacteria that have lysed by assuming that the average yield per bacterium is the same for bacteria lysing early or late, which is by no means certain.

TABLE 2
Action of completely inhibitory concentrations of proflavine

Proflavine in C.I. concentration was added to bacteria 2 minutes before adding phage. After 7 to 10 minutes phage adsorption was interrupted by a dilution of 1:2,000 in proflavine medium containing the C.I. concentration. Platings were made at intervals. The data in columns 2 and 4 were obtained in parallel experiments for T6 and T7. For T2, they were from different experiments. The control value represents the phage input in the absence of proflavine.

TIME AFTER IN-FECTION, MINUTES	NO PROFLAVINE		C.I. CONCENTRATION OF PROFLAVINE	
	Plaque count at time indicated	% rise completed	Plaque count at time indicated	% loss adsorbed phage
T2 (latent period 21 minutes, C.I. concentration 0.35 mg/100 ml)				
8	7.8×10^6	0	2.7×10^6	0
14	7.6×10^6	0		
21	9.9×10^6	0.55	2.9×10^6	0
24	1.3×10^7	1.3		
27	1.2×10^8	28		
30	2.8×10^8	68	1.1×10^6	71
40	3.8×10^8	93	8.1×10^5	85
50	4.0×10^8	100	3.4×10^5	100
Free			5.0×10^5	
Control			2.9×10^6	
T6 (latent period 30 minutes, C.I. concentration 0.05 mg/100 ml)				
12	6.04×10^6	0	6.74×10^6	0
25	5.52×10^6	0	7.16×10^6	0
30	6.4×10^6	0	6.36×10^6	0
33	8.0×10^6	0.2	5.82×10^6	15
35	1.6×10^7	1	4.44×10^6	39
37			3.5×10^6	55
39	6.2×10^7	6	2.36×10^6	75
41	2.1×10^8	22	1.92×10^6	82
43	3.1×10^8	33	1.66×10^6	86
45	3.8×10^8	40	1.6×10^6	87
47	4.9×10^8	52	1.22×10^6	94
49	5.6×10^8	60	1.08×10^6	96
55			9.6×10^6	98
60	7.7×10^8	83		
65	8.1×10^8	86	8.0×10^5	100
75	9.3×10^8	100	6.2×10^5	
90	9.2×10^8		5.0×10^5	
Free			8.6×10^5	
T7 (latent period 15 minutes, C.I. concentration 0.75 mg/100 ml)				
9	5.6×10^6	0	4.4×10^6	0
18	1.4×10^7	12	4.3×10^6	7
23	4.0×10^7	47	3.5×10^6	60
30	6.9×10^7	87	3.2×10^6	80
40	7.3×10^7	100	2.8×10^6	100
50	7.2×10^7		2.0×10^6	
Free			2.9×10^6	

without releasing any phage. Representative experiments presented in table 3 show that the anticipated results were obtained. With T2, the yield is reduced by 61 per cent for dilution at 30 minutes and by 96 per cent for dilution at 40 minutes. With T6, the reduction in yield is 34 per cent at 39 minutes and 79 per cent at 55 minutes. This emphasizes the correlation of the longer latent period for T6 with the longer time required before phage is lost in the presence of proflavine.
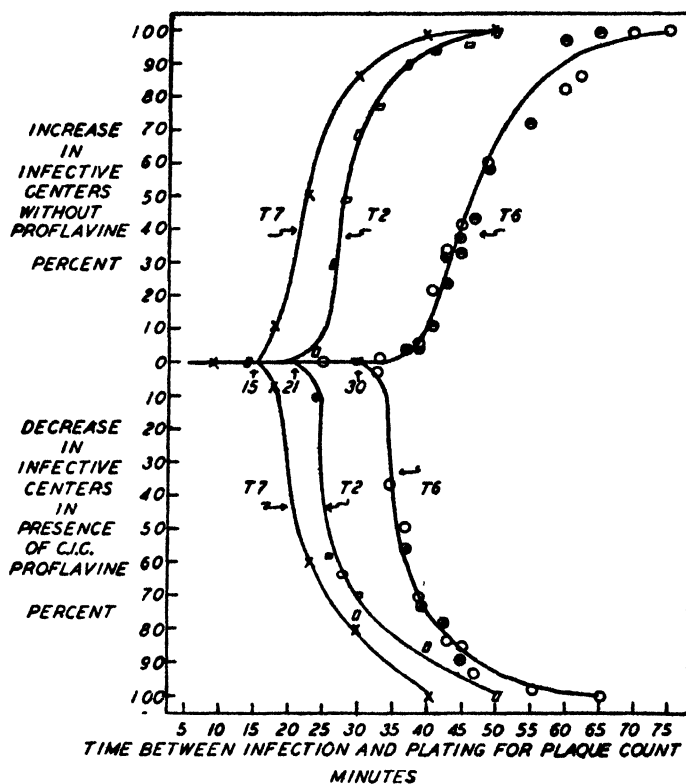
Figure 1. Correlation between loss of infective centers in the presence of the C.I. concentration of proflavine and liberation of phage in its absence. Results for phages T2, T6, and T7 are presented. Note that the first loss of infective centers occurs at the end of the latent period, and that complete loss is observed at the end of the normal rise period for each phage type.

We may then conclude that, in the presence of the C.I. concentration of proflavine, the phage inside the infected cells is not lost until a time shortly before—or corresponding to the time of—the lysis of the host. Until this time phage can be liberated if proflavine is removed. Lysis in the presence of proflavine does not produce active phage, and even the original infecting particle is lost.

It was possible that bacteria lysed in the presence of proflavine, although releasing no active phage, might liberate particles that could be adsorbed by host cells

but that failed to multiply. Since adsorption of phage is generally followed by death of the host cell, we attempted to detect host-killing activity in proflavine lysates. Parallel bacterial cultures—one with and the other without proflavine —were inoculated with phage, incubated for 2 hours, and filtered. They were tested, immediately and after storage, for plaque production and bactericidal

TABLE 3

Phage liberation after removal of completely inhibitory concentrations of proflavine by dilution

The technique is the same as described in table 2 with additional dilutions to remove proflavine. These dilutions were 1:20 for T2 and T7 and 1:50 for T6. The final titers were determined at the times indicated in the table. The control values represent the yield in the absence of proflavine in a parallel growth tube. The lower control value for T7 is characteristic.

TIME OF REMOVAL FROM PROFLAVINE (MINUTES AFTER INFECTION)	FINAL TITER FOLLOWING DILUTION AT TIME INDICATED	YIELD AFTER DILUTION IN PERCENTAGE OF MAXIMUM YIELD	TIME OF ASSAY (MINUTES AFTER INFECTION)
T2 (latent period 21 minutes)			
10	1.4×10^8	100	57
20	1.4×10^8	100	59
30	5.4×10^7	39	65
40	5.2×10^6	4	75
50	1.2×10^6	0.9	85
Free	4.0×10^5		
Control	1.7×10^8		55
T6 (latent period 30 minutes)			
10	3.2×10^8	100	67
20	3.3×10^8	100	69
30	3.0×10^8	94	71
39	2.1×10^8	66	75
55	6.6×10^7	21	85
65	2.6×10^7	8	95
Free	1.4×10^6		
Control	5.1×10^8		65
T7 (latent period 15 minutes)			
11	1.5×10^8	100	54
18	1.1×10^8	73	56
23	9.4×10^7	63	58
30	2.9×10^7	19	60
40	6.4×10^6	4	62
Free	4.5×10^6		
Control	7.2×10^7		50

activity. All bactericidal activity could be accounted for by the active phage present. This does not exclude the possibility that bacteria lysed in proflavine liberate some kind of inactive, nonbactericidal phage particle.

The time of occurrence of the proflavine-inhibited reaction. We have seen that, in the presence of proflavine, ability to liberate phage is not lost until approxi-

mately the time when the infected bacterium is lysed. This may indicate either that the infecting particle remains, as it were, in a dormant state, as found for 5-methyl tryptophan (Cohen and Anderson, 1946; Cohen and Fowler, 1947), or that the processes leading to phage multiplication go on inside the bacterium but that some late or even final reaction needed for production of active phage is inhibited. This point was tested by determining the length of the interval between dilution in M-9 medium to remove proflavine and the initiation of the rise in titer. The results shown in table 4 indicate that phage liberation begins almost immediately upon dilution and proceeds at a normal rate.

TABLE 4

Absence of latent period after removal of proflavine

The procedure was the same as described in table 2 with an additional dilution 1:20 from proflavine at the times indicated in column 1. The titer of this growth tube was then determined at the times specified in column 2.

TIME OF REMOVAL FROM PROFLAVINE (MINUTES)	TIME OF ASSAY (MINUTES AFTER REMOVAL FROM PROFLAVINE)	TITER
T2 (latent period 21 minutes)		
28	0	3.8×10^6
	3	1.1×10^7
	5	$>4.0 \times 10^7$
40	0	2.7×10^6
	2	3.2×10^6
	4	4.3×10^6
	6	1.1×10^7
	8	2.0×10^7
T6 (latent period 30 minutes)		
34	0	1.2×10^7
	4	1.9×10^7
	6	2.2×10^7
	8	4.1×10^7
	11	2.2×10^8
	16	7.9×10^8
	31	1.5×10^9

We may then conclude that some of the reactions leading to phage production proceed in the presence of proflavine, and that the inhibitory action is exerted on some late step in the process. If so, the addition of proflavine at various intervals after infection should only suppress phage growth to the extent that the reactions inhibited by proflavine have not yet taken place. Several experiments of this type were performed. Table 5 shows the results of two representative experiments, in which the C.I. concentration of proflavine was added at various intervals after infection and all tubes were assayed at a time when the rise in the control tube had been completed. It will be seen that, both with T2 and T6, by

17 minutes the process of phage formation has already reached a stage at which some active phage can be produced in the presence of proflavine. A progressive

TABLE 5

Addition of the C.I. concentration of proflavine at various intervals after infection

The procedure \. as that of the one-step growth experiment. The mixture of phage and bacteria was diluted after 8 minutes and divided into a series of tubes, one of which received the C.I. concentration of proflavine at one of the times indicated in column 1. The yield of phage in the presence of proflavine was measured between 52 and 60 minutes for T2, and between 60 and 70 minutes for T6. The value for the time "−2 minutes" was obtained by a parallel experiment similar to the one of table 2.

TIME BETWEEN INFECTION AND ADDITION OF PROFLAVINE (MINUTES)	FINAL TITER AFTER LYSIS	YIELD IN PERCENTAGE OF YIELD IN CONTROL
T2 (latent period 21 minutes). Input 1.6 × 10⁷		
−2	2.0×10^6	0
10	1.9×10^6	0
16	1.2×10^8	12
20	3.5×10^8	35
24	7.7×10^8	77
28	8.1×10^8	81
32	8.9×10^8	89
36	9.3×10^8	93
40	9.2×10^8	92
44	9.7×10^8	97
No proflavine	1.0×10^9	100
T6 (latent period 30 minutes). Input 5.2 × 10⁶		
−2	4.2×10^5	0
15	3.4×10^6	0.8
17	9.1×10^6	2.3
20	2.8×10^7	7
25	9.1×10^7	23
30	2.9×10^8	74
35	3.3×10^8	85
40	4.2×10^8	108
45	4.5×10^8	115
50	4.8×10^8	123
No proflavine	3.9×10^8	100*

*Since the yield in the control tube for this experiment was abnormally low, in plotting these results in figure 2 we used the values as percentages of the maximum titer reached with proflavine input at 50 minutes. The apparent increase in yield over the control is not significant.

increase in phage production is found as proflavine is added at later times. Ex-trapolation from these and other data not included in the table suggests that the latest input time for complete suppression is about 12 to 15 minutes after infec-

tion, this time being possibly earlier for T2 than for T6. The results of experiments of this type are plotted in figure 2, which shows the progressive increase in phage liberation as a function of the time of addition of proflavine.

The data in table 5 and figure 2 may be interpreted as suggesting that the particles inside the bacteria reach at various times a stage at which proflavine can no
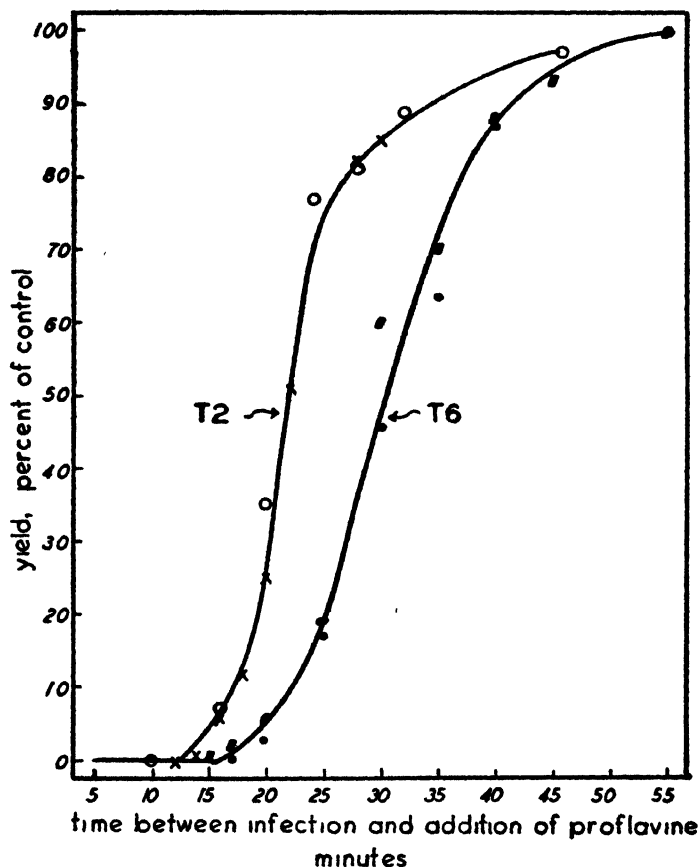
Figure 2. Yield of active phage (measured at the end of the normal rise period) when proflavine is added at different times after infection. No viable phage is produced if the drug is added during the first 12 to 15 minutes after infection. If proflavine is added later, the yield progressively increases with the interval between infection and the addition of the drug.

longer prevent the further steps necessary to produce viable phage. They may, therefore, give some information on the rate of phage growth. Luria and Latarjet (1947), using ultraviolet light to inactivate phage T2 during intracellular growth, found that a multiple-hit curve begins to appear between 7 and 12 minutes after infection, from which they concluded that, in the multiplication of this phage, the number of particles begins to increase at this period. Confirmation of this conclusion appears to be provided by the use of X-rays (Latarjet,

1948). Although these observations may support the hypothesis that by the addition of proflavine at intervals after infection we can measure the actual increase in phage within the culture during the latent period, our data are insufficient to justify a quantitative analysis of this type.

The fact that no phage liberation occurs if proflavine is added before a critical time in the latent period suggests the possibility that no fully active phage is present as such in the cell up to that time. This suggestion is in agreement with the recent findings of Luria (1947) and Doermann (private communication). The stage in phage production blocked by proflavine may be connected with the appearance of fully active phage particles.

The slow rise in phage yield as a function of the time of input of proflavine (figure 2) might be due to one or two reasons: (1) the sequel of reactions involved in phage production reaches, earlier in some cells than in others, a stage that can no longer be blocked by proflavine, just as some cells lyse before others; or (2) the processes leading to the production of individual phage particles inside each cell reach at different times a stage that can no longer be blocked by proflavine, there being in each cell a gradual accumulation of phage particles whose production has reached this stage. This hypothesis does not in itself imply any specific mechanism of phage reproduction.

In an attempt to distinguish between these two possibilities, single burst experiments were performed, adding proflavine at different times during the latent period before the samples were diluted in proflavine and distributed to individual tubes. All tubes were plated at the time at which the rise period in the absence of proflavine was completed. The number of cells yielding active phage in the presence of proflavine can be obtained directly from the number of samples without bursts. If the fraction of samples without bursts is e^{-x}, then the number of bursts per sample is given by x. By the first hypothesis—difference in critical stage among bacterial cells—a few tubes should show a normal burst, but the others should show no phage. By the second hypothesis—difference in critical stage among phage particles—many tubes should show a burst, but this burst should be small. Only one single-burst experiment was done for each time of proflavine addition. Table 6 shows the results of these experiments for T2 and T6. Although more experiments would be desirable, a tentative conclusion seems justified.

With both phages the fraction of infected cells capable of liberating phage in the presence of proflavine (table 6, column 8) increases rapidly, but the average yield per fertile burst (table 6, column 10) is much lower than the average yield per burst in the control, even when a majority of the infected cells already give active phage in the presence of proflavine. Both the number of bacteria that liberate phage and the amount of phage liberated per bacterium increase as the time of the addition of proflavine approaches the end of the latent period. Since we have already concluded that the inhibitory action of proflavine is exerted on some late step in the process of phage production, we incline to the belief that individual phage particles reach at various times the stage which is blocked by proflavine, and that a bacterium liberates active phage in amounts corresponding

to the number of particles that had completed this stage at the time of the addition of proflavine.

The effect of addition of proflavine to bacteria prior to infection with T2. In the experiments discussed above proflavine was added to bacteria 2 minutes before adding the bacteriophage. When the host, however, is exposed to the C.I. concentration of proflavine for longer periods of time before infection with T2 (45 minutes to 4 hours) and the phage is then added and liberation tested at intervals, some phage production occurs in the presence of the C.I. concentration, the more so the longer the time during which the bacteria have been in contact with the drug. The effect is not due to reduction in drug concentration by previous

TABLE 6

Single burst experiments: proflavine added at intervals during the latent period

Bacteria were mixed with phage in the absence of proflavine. Eight minutes later, the mixture was diluted in M-9 medium to stop adsorption. At the time indicated in column 2, the C.I. concentration of proflavine was added to the diluted mixture. The mixture was further diluted in medium with the C.I. concentration of proflavine in such a way that, when distributed in single-drop samples to a large number of tubes, it would give a suitable number of bursts per tube. The final plating of the entire contents of each tube was done at the time of the maximum titer in the control.

PHAGE TYPE	TIME BETWEEN INFECTION AND ADDITION OF PROFLAVINE (MINUTES)	NUMBER OF SAMPLES	INFECTED BACTERIA IN ALL SAMPLES	INFECTED BACTERIA PER SAMPLE	SAMPLES WITHOUT FERTILE BURSTS	PROPORTION OF SAMPLES WITHOUT FERTILE BURSTS, e^{-s}	AVERAGE NUMBER OF BURSTS PER SAMPLE, s	INFECTED BACTERIA YIELDING PHAGE (%)	TOTAL YIELD IN ALL SAMPLES MINUS FREE PHAGE	AVERAGE YIELD PER FERTILE BURST	RANGE OF YIELDS IN INDIVIDUAL FERTILE BURSTS
T2	16	36	236	6.56	6	0.167	1.79	27	387	6.0	3–35
	20	36	140	3.9	3	0.083	2.48	63.7	1,328	14.9	3–114
	No proflavine	36	59	1.64	7	0.194	1.64	100	2,066	35	5–367
T6	20	24	34	1.42	16	0.666	0.41	28.8	148	15.1	2–44
	27	36	23.5	0.65	19	0.5277	0.64	98	671	29.1	3–82
	No proflavine	24	20.9	0.87	10	0.417	0.87	100	2,045	98	48–322

contact with bacteria, since all samples are diluted in fresh medium containing the C.I. concentration 8 minutes after infection. The reduction in inhibitory effect of proflavine after prolonged exposure of the bacteria is even more pronounced when tested with dilution from proflavine 35 minutes after infection.

Although proflavine-resistant bacterial mutants can be obtained, the phenomenon discussed here was shown not to be due to a selection of mutants. We incline to the belief that during growth in the presence of proflavine an adaptation of the host occurs, which makes the cells more suitable for phage growth. This may be caused, either by the accumulation during this period of some material needed for phage growth, or by the activation of an alternate metabolic pathway removing the block to phage production (for adaptation to proflavine see Hinshelwood, 1946).

Bacteriophage mutants resistant to proflavine. By plating large amounts of the most sensitive phage types, T4 and T6, on agar containing inhibitory concentrations of proflavine a few plaques were obtained. Stocks prepared from these plaques in medium without proflavine showed higher proflavine tolerance than the respective wild type. The process could be repeated, with further increase in tolerance. The danger of contamination of the stocks with other phages used in the laboratory was eliminated by sensitivity tests with a number of indicator bacterial strains (Delbrück and Luria, 1942). The differences in proflavine tolerance between mutants and wild types, as measured by the C.I. concentrations, were significant. A threefold increase in resistance was observed for T6, and a twofold increase for T4.

These experiments, by showing that proflavine sensitivity of a phage can be brought about by a heritable change in the phage, suggest that some of the mechanisms of intracellular growth which are prevented by proflavine may be carried by the phage particle itself.

Antagonism of proflavine inhibition of T2 multiplication. In addition to ribose nucleic acid, polymerized and depolymerized desoxyribose nucleic acid (DNA)[5] were tested as antagonists of proflavine. Complete reversal of inhibition of phage growth was obtained with both preparations of DNA at 0.25 mg per 100 ml. The ribose nucleic acid was slightly less effective. Casein hydrolyzate or riboflavin produced very little reversal. Cohen (1948) measured the increase in DNA in bacteria following infection with bacteriophage T2, and found that increase in DNA began several minutes after infection. It seems possible that the rise in DNA may parallel the appearance of phage that has completed the proflavine-inhibited step, and that the chemical step subject to proflavine inhibition is related to the formation or utilization of DNA for phage synthesis (McIlwain, 1941).

DISCUSSION AND SUMMARY

The use of proflavine as an inhibitor of phage growth has demonstrated that the characteristics both of the host and of the phage affect the response of infected bacteria to the drug. On the one hand, adaptation of the host may permit phage growth in the presence of the drug. On the other hand, the growth sensitivity to proflavine is characteristic of each phage type and can be altered by mutation of the phage. Confirming the observation of Fitzgerald and collaborators (1946) we found that the growth of several phages, particularly those of the "large particle" type, is completely inhibited by drug concentrations that allow normal or subnormal multiplication of the host.

Proflavine appears to block a late reaction necessary for the production of active phage, whereas some of the earlier processes leading to phage production are completed in the presence of the drug, since the removal of proflavine allows phage liberation without any appreciable delay. Phage-infected bacteria in which the production of phage is inhibited by proflavine are lysed after a normal

[5] Kindly supplied by Dr. Seymour S. Cohen.

latent period, but fail to liberate any active phage particles. In these respects, the inhibition by proflavine differs from the inhibition of T2 growth by 5-methyl tryptophan (Cohen and Anderson, 1946; Cohen and Fowler, 1947), which appears to interrupt phage growth completely. It is not known whether or not infected bacteria inhibited by 5-methyl tryptophan undergo lysis, like those inhibited by proflavine. Particularly in view of the different modes of action of the two inhibitors, it is interesting that in both cases complete inhibition can be obtained only if the inhibitor is introduced within 12 to 14 minutes after infection. It may be that this represents the minimum time of appearance of active phage particles inside the bacterium. It is possible that the proflavine-blocked reactions, which we have shown to take place relatively late in the course of infection, involve the formation or utilization of desoxyribose nucleic acid, the synthesis of which has been found (Cohen, 1948) to begin several minutes after infection with bacteriophage.

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MECHANISM OF PROPIONIC ACID FORMATION BY PROPIONIBACTERIUM PENTOSACEUM

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Decarboxylation of succinic acid as a mode of formation of propionic acid in the genus *Propionibacterium* has been suggested (Werkman and Wood, 1942), and evidence has been presented that supports this contention. Numerous observations concerning the ability of the genus to ferment succinic acid with the formation of propionic acid have been made (Shaw and Sherman, 1923; Hitchner, 1934; Fromageot and Bost, 1938). Wood and Werkman (1942) determined the products of the anaerobic breakdown of succinic acid and reported the production of propionic acid and carbon dioxide in approximately equimolar amounts along with small amounts of acetic acid. They reported the production of no other products. The data obtained with isotopic carbon by Carson and Ruben (1940) and Wood *et al.* (1940, 1941) constitute evidence that propionic acid results from the decarboxylation of a symmetrical dicarboxylic acid.

A recent report of investigations by Johns (1948) indicates that bacterial decarboxylation of succinate as a major reaction has been observed with an anaerobic micrococcus isolated from the rumen of sheep. This organism was reported as being strictly anaerobic and differing from the propionic acid bacteria in that it did not ferment sugars. Optimum pH for the decarboxylation was reported as 7.4, which is much higher than the optimum pH of 5.1 to 5.2 of the succinic decarboxylase system described in this paper.

No adequate data, however, have been available showing that the genus *Propionibacterium* can decarboxylate succinic acid at a rate which can account for this pathway as a principal mechanism. Data are herewith presented which demonstrate that succinic acid can be decarboxylated at a rate which can account for all of the propionic acid produced from pyruvic acid under similar conditions.

EXPERIMENTAL METHODS

Cultural methods. Strain E214 of *Propionibacterium pentosaceum* of Professor C. B. van Niel's collection was obtained through the courtesy of Dr. Fritz Lipmann and was used in all experiments.

Cultures were grown for 36 hours at 30 C after a 5 per cent inoculation into a 4-liter Erlenmeyer flask containing 2 liters of a medium composed of 0.5 per cent each of glucose, peptone, and Difco yeast extract. At the end of the incubation period the cells were removed by centrifugation, washed twice in a volume of distilled water equal to the original volume of the fermentation broth, and resuspended in phosphate buffer of pH 5.2 or in distilled water.

Manometric methods. All of the experimental work concerning rates of fer-

mentation of pyruvate, succinate, and other substrates was conducted by means of the usual Warburg manometric techniques.   A total of 3.0 ml was contained in each Warburg vessel and was composed of 1.0 ml of a cell suspension, 0.2 ml of substrate in the side arm, and 0.3 ml of semicarbazide when this latter substance was included.   The total volume was made up to 3.0 ml by the addition of phosphate buffer.   Specific details concerning cell, buffer, substrate, and inhibitor concentrations and pH are given under "Experimental Results" for each individual experiment.   All manometric experiments were conducted at 30 C under an atmosphere of nitrogen.

In earlier experiments when the activities of dried cell preparations were tested, the drying was done by placing the freshly harvested and washed cells in 2- to 3-millimeter layers in petri dishes and drying rapidly *in vacuo* over "drierite." The dried bacteria were stored at 5 C.   For use a weighed quantity of cells was resuspended in $M/100$ phosphate buffer to the desired concentration.

Analytical methods.   Volatile acids were determined by pooling several Warburg cups, or by taking an aliquot of the fermentation broth, and proceeding according to the general distillation and partition methods described by Osburn, Wood, and Werkman (1936).   When volatile acids were determined from pooled Warburg vessel contents, microtitration methods were employed, which enabled an accuracy of measurement of 0.02 ml of the titration alkali.   Molar percentages of acetic and propionic acids were determined from a standard curve carefully worked out with pure acetic and propionic acids of a concentration of the order of the samples to be analyzed.

The partition method of analysis is based upon the distribution of two acids between two immiscible solvents, such as water and diethyl ether, when dilute aqueous solutions of the acids are shaken with acid-free ether in separatory funnels.   Each acid of a pair of acids will show a characteristically different distribution, which may be quantitatively determined by titration of the aqueous layer. In the experimental work here reported this principle of distribution between two phases was reduced to an analytical basis as follows: 25 ml of a solution of propionic acid of a known concentration were shaken in a separatory funnel with 50 ml of acid-free ether.   The aqueous layer was removed and titrated with 0.01 N NaOH, and this value was designated as N_2.   An aliquot of the same acid solution was titrated with no previous ether extraction.   This titration value was designated as N_1.   The "partition constant," designated as K_1, was obtained by dividing N_2 by N_1 and multiplying by 100.   A similar procedure was followed with a solution of acetic acid of equimolar concentration.   A "partition constant" for acetic acid was thus obtained.   Plotting the K_1 values on the ordinate and molar percentages on the abcissa, a standard curve was established with the K values of pure solutions of acetic and propionic acids providing the minimal two points.   Mixtures of the two acids of known concentrations were treated in an identical manner, and it was found that the K values thus obtained fell on the straight line already established with the K values of the 100 molar per cent acids.   An identical procedure was followed with different proportions of acid solution and ether.   Twenty-five ml of aqueous acid phase and 25 ml of ether

phase were used in this second standardization. "Partition constants" of a different magnitude resulted, and a different standard curve was drawn. Unknown acid solutions were analyzed by obtaining the K_1 and K_2 values by the method described, and reading molar concentrations from the standard curves. The presence of a third acid or of any volatile neutral product would have been reflected by poor agreement of the analytical data from both K_1 and K_2 curves. Analytical data were not accepted as final if poor agreement from K_1 and K_2 values was obtained.

When pyruvate was present in the fermentation mixture, it was removed by shaking for at least 10 hours with 2,4-dinitrophenylhydrazine under acidic conditions.

Of the chemicals used, only the semicarbazide needs special mention. In order to perform the analytical techniques, no volatile acid of a foreign nature could be present; hence the hydrochloride could not be used. Chemically pure semicarbazide was converted to the sulfuric acid salt by digesting with a slight excess of an equivalence of silver sulfate and subsequently removing the excess silver ion by precipitation with hydrogen sulfide. The resulting chloride-free solution was boiled to remove dissolved hydrogen sulfide, adjusted to pH 5.2, and then made up to volume.

EXPERIMENTAL RESULTS

Comparative rates of decomposition of pyruvate, succinate, fumarate, and L-malate were obtained manometrically. Each vessel contained 1.0 ml of a suspension of cells in M/100 phosphate buffer of pH 5.2, and 0.2 ml of M/5 substrate in the side arm. Semicarbazide was included when succinate, fumarate, and L-malate were the substrates, and was added in the amount of 0.3 ml of M/10 semicarbazide sulfate prepared as described under "Experimental Methods." The total volume in all cases was made up to 3.0 ml by the addition of M/100 phosphate buffer of pH 5.2. All solutions were at pH 5.2 before addition to the Warburg cup. Incubation was at 30 C for 120 minutes under an atmosphere of nitrogen. Activity was expressed as μL of CO_2 per mg per hour, hereafter designated as Q_{CO_2}.

Both pyruvate and succinate were found to be decomposed at significant rates (Q_{CO_2} of 11.1 and 4.4, respectively, for the first 60 minutes when 35 mg of cells were present); fumarate was decomposed significantly rapidly (Q_{CO_2} of 2.9); and L-malate was decomposed at a slow rate (see figure 1). Of significance in the case of fumarate decomposition is the observation that with a decreased level of cells present (10 mg per cup) the relative rate of decomposition of fumarate compared to succinate is greatly reduced (figure 2). Making the reasonable assumption that it is the succinate that is being decarboxylated, this difference can be explained on the basis of the greater reducing capacity of the higher concentration of cells. The activity on the L-malate was quite logically found to be lower than the activity of the fumarate and was also decreased by the presence of fewer cells. Semicarbazide was included as a ketone-trapping agent to eliminate the possibility of reversion to pyruvate in the cases of the dicarboxylic acids. This
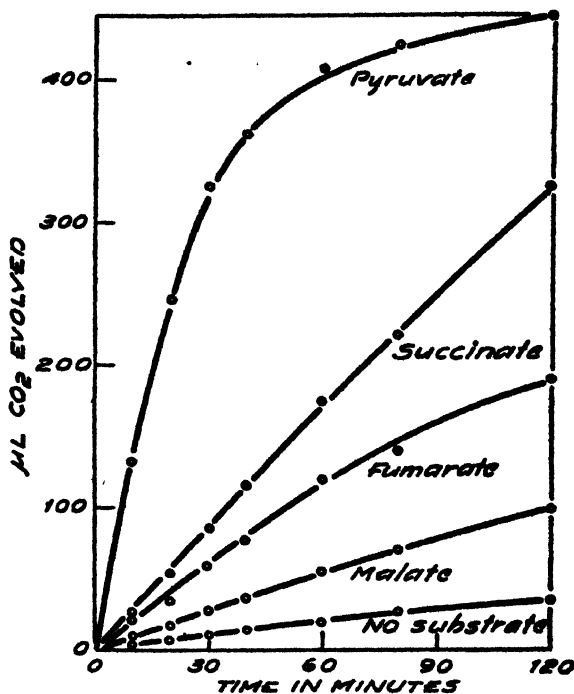
Figure 1. Activity of heavy cell suspension (35 mg per vessel).
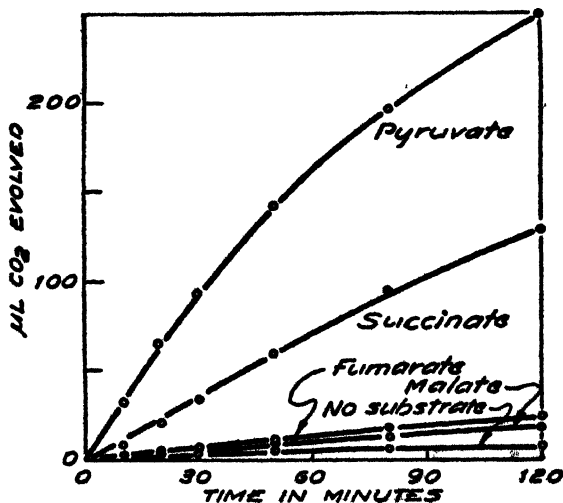
Figure 2. Activity of light cell suspension (10 mg per vessel).

addition can be considered as a mere precautionary measure, since separate experiment established that the rate of decomposition of succinate, fumarate, and malate remained unchanged upon the addition of this inhibitor.

A similar experiment was conducted in order to determine the amounts and proportions of volatile acids produced and the carbon dioxide evolved from pyruvate as compared to succinate. The experiment was identical to the one concerning rates with the exception that the incubation period was increased to 240 minutes and the only vessels utilized were six with pyruvate as a substrate and six with succinate as a substrate. In each case six control cups were run for the purpose of making endogenous corrections of the analysis data. At the end of the incubation period 0.5 ml of 5 N H₂SO₄ were added to each cup to stop the fermentation reactions, and the six cups were pooled for analysis. They were distilled and analyzed for volatile acids by the partition methods described. For amounts and proportions of volatile acids and CO₂ produced, refer to table 1.

In the experimental work which resulted in the data presented in table 1, the same crop of cells was used in all fermentations, and identical experimental conditions were maintained; hence, the data may be considered completely comparable. Of primary significance is the observation that in the decomposition of succinate,

TABLE 1

Volatile acids and carbon dioxide produced from pyruvate and succinate under identical conditions

	FROM PYRUVATE*	FROM SUCCINATE*
Acetic acid......................................	114 μ Moles	6 μ Moles
Propionic acid.............................	46 μ Moles	96 μ Moles
Carbon dioxide............................	129 μ Moles	108 μ Moles

* Values are corrected for the following endogenous production: 73 μM acetic acid, 38 μM propionic acid, and 34 μM CO₂. Endogenous values remained the same in the presence of semicarbazide.

propionate, appearing quite logically in approximately equimolar amounts with carbon dioxide, is produced in significantly greater amounts than in the case of pyruvate decomposition.

Preliminary studies of the succinic decarboxylase system have shown it to be most active at pH 5.1 to 5.2 (figure 3), as previously reported by Werkman and Wood (1942). In determining the optimum pH, manometric measurements of carbon dioxide evolution were made. Each Warburg cup contained 1.0 ml of a suspension of 10 mg of washed cells suspended in distilled water, 0.2 ml of M/5 succinate in the side arm, and 1.8 ml of M/100 phosphate buffer. The latter two constituents were adjusted to the desired pH values before addition to the cup. A vessel without substrate was included for each pH value at which utilization was tested. Each of the points indicated on the curve in figure 3 is properly corrected for the endogenous fermentation. Incubation was made at 30 C for 1 hour under an atmosphere of nitrogen. Activity in terms of Q_{CO_2} was determined and plotted against pH values (figure 3).

The activities of cell preparations in the decarboxylation of succinate from cultures ranging in age from 12 hours to 16 days were determined by the manometric

method described in the rate studies. It was found that activity was greatest after 32 to 38 hours of incubation. After 6 days the activity had dropped to one-tenth, and it remained at about the same low value for cultures ranging in age up to 11 days. After 15 days of incubation activity was insignificant.

A dried preparation of *Propionibacterium pentosaceum* was examined for activity on succinate. No significant activity was obtained with this preparation.
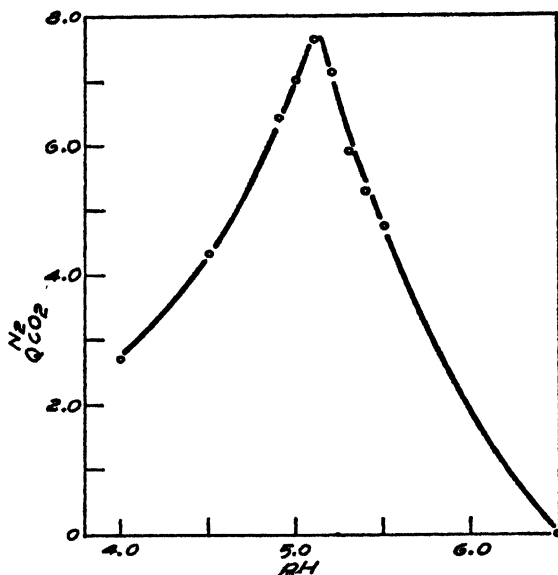
Figure 3. Effect of pH upon the activity of the succinic decarboxylase system.

TABLE 2

The production of volatile acids from glucose as related to the pH of a buffered culture medium

TIME OF INCUBATION	pH	μM ACETIC ACID	μM PROPIONIC ACID
19 hours	7.12	39	0
23 hours	6.76	160	111
25 hours	6.45	168	201
27 hours	5.93	225	219
29 hours	5.21	234	365
31 hours	4.80	244	524
41 hours	4.75	269	634

A study of the production of acetic and propionic acids as related to pH, and therefore to the activity of the succinic decarboxylase system, was made by the analysis of an ordinary fermentation broth for volatile acids at varying time and pH intervals throughout the fermentation. Aliquots of an ordinary culture medium composed of 0.5 per cent each of glucose, peptone, Difco yeast extract, and K_2HPO_4 were analyzed at such intervals as to cover the critical pH range of an assumed succinic decarboxylase system. In general, it was apparent that

significant amounts of propionic acid did not appear until the pH had dropped below 6.5, and that peak production did not appear until the pH was well below 6.0. Careful examination of table 2, however, will show that somewhat contradictory to the general tenets already set forward is the appearance of appreciable amounts of propionic acid at a pH of 6.76, whereas studies concerning optimum activity of the succinic decarboxylase system indicate only slight activity at pH 6.5. This apparent discrepancy is not necessarily evidence for an alternate system of propionate formation. All experimental work was conducted with intact cells in which cell permeability is a decided factor in the utilization of any substrate. It is not unreasonable to visualize that at higher pH ranges the cell membranes may not be permeable to succinate, a situation which, with growing cells, would not be a factor since the succinate would necessarily be produced within the boundaries of the cell.

Studies concerning the optimum pH of the succinic decarboxylase system show greatest activity at the general pH range of 4.5 to 5.5, with slight activity as high as pH 6.5. Thus it is evident that in ordinary culture media the production of propionic acid occurs at a pH range not incompatible with the functioning of a succinic decarboxylase system as at least an important mechanism.

In studies of this nature it is desirable to separate and delineate critical reactions insofar as possible. The use of malonate in a concentration of 0.3 M in the Warburg cup was found to cause an approximately 90 per cent inhibition of the functioning of the decarboxylase on succinate. If succinate decarboxylation was the main pathway to propionic acid, it would then have to follow that, in a system inhibited by malonate, propionate production from pyruvate would be essentially eliminated. Using the methods already described in the work concerning the amounts and proportions of volatile acids produced, this hypothesis was tested for experimental validity. When the contents of the six pooled cups were analyzed, it was found that in the presence of 0.3 M malonate only traces of propionate were produced from pyruvate.

DISCUSSION

The formation of propionate from pyruvate can be postulated as including the following major reactions resulting in the over-all transformation indicated by reaction (7):

(1) $2CH_3COCOOH + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H$

(2) $CH_3COCOOH + CO_2 \rightarrow COOHCH_2COCOOH$

(3) $COOHCH_2COCOOH + 2H \rightarrow COOHCH_2CHOHCOOH$

(4) $COOHCH_2CHOHCOOH \rightarrow COOHCH{=}CHCOOH + H_2O$

(5) $COOHCH{=}CHCOOH + 2H \rightarrow COOHCH_2CH_2COOH$

(6) $COOHCH_2CH_2COOH \rightarrow CH_3CH_2COOH + CO_2$

(7) $3CH_3COCOOH + H_2O \rightarrow 2CH_3COOH + CH_3CH_2COOH + 2CO_2$
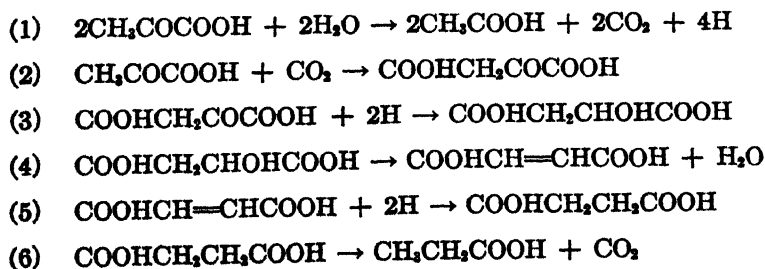
Evidence in favor of such a scheme is not lacking. The carbon dioxide fixation step is supported by the original studies of Wood and Werkman (1936a) on the general problem of heterotrophic carbon dioxide fixation and by their more specific investigations (1936b, 1938, 1940). The investigations with carbon isotopes conducted by Carson and Ruben (1940) and Wood et al. (1940, 1941) support a theory of random decarboxylation of a symmetrical dicarboxylic acid in the formation of propionate from glycerol and isotope-labeled carbon dioxide. The series of reactions from oxalacetate to succinate have been shown by Krebs and Eggleston (1941) to be reversible in the case of the propionic acid bacteria.

The possibility of the decarboxylation of succinic acid as a major reaction in the formation of propionic acid was considered by Werkman and Wood (1942). In their review concerning heterotrophic assimilation of carbon dioxide, they present an excellent analysis of the isotope data previously mentioned, and in their treatment of the subject they state: "This is further evidence that the propionic acid is formed in the glycerol fermentations exclusively by decarboxylation of a symmetrical dicarboxylic acid. It is the mechanism of the decarboxylation that particularly is uncertain." Also: "There is some evidence that the propionic acid bacteria can decarboxylate succinate anaerobically ($COOHCH_2CH_2COOH \rightarrow CO_2 + CH_3CH_2COOH$) but it is questionable whether or not the rate of this reaction is high enough to be of any considerable importance." They describe unpublished experiments in their laboratory in which *Propionibacterium arabinosum* fermented succinate to propionic acid, carbon dioxide, and small amounts of acetic acid, but a large conversion was not obtained.

Noteworthy are the earlier investigations of Shaw and Sherman (1923), Hitchner (1934), and Wood et al. (1937) in which the fermentation of succinate to propionate is reported. Although in all cases these investigations were in the nature of fermentation studies and the more controlled conditions of resting cell techniques were not employed, their data in the light of the more recent isotope studies and the findings reported in this paper become more meaningful.

Fromageot and Bost (1938) made the interesting observation that their culture of *Propionibacterium pentosaceum* could produce propionic acid from succinate only in the presence of glucose. They worked with 48-hour wet cell suspensions in a pH 6.4 bicarbonate buffer. An explanation of their failure to show activity on succinate alone could be the unfavorable pH. When glucose was present, acid production could occur to an extent that could easily lower the pH sufficiently to permit activity of a succinate decarboxylase of the type described in this paper.

Acetate condensation as a mode of formation of succinate is under investigation by Carson et al. (1948). In abstracted form they suggest that a C_1 condensation may occur. The analytical data of table 1 do not support a theory of acetate condensation as a major mechanism.

The possibility of the direct reduction of pyruvate with lactate as an intermediate has been well eliminated by the fluoride inhibition studies of Chaix-Audemand as cited by Barker and Lipmann (1944), and also by the more detailed investigations of the latter. With the revision of the hypothetical scheme of Barker and Lipmann (1944) to include a carbon dioxide fixation step and the

necessary reductions, succinate can be included as the hypothetical compound "X." The fluoride sensitivity they discuss is probably concerned in a lactate to pyruvate transformation and is possibly a matter of inhibition of a hydrogen transport mechanism.

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. W. W. Umbreit of the Merck Institute and to Professor James M. Sherman for their many invaluable suggestions throughout the course of this investigation.

SUMMARY

Data are presented which demonstrate that *Propionibacterium pentosaceum* possesses a succinic decarboxylase system sufficiently active to produce propionic acid from succinic acid at a rate comparable to the rate of production of propionic acid from pyruvic acid.

The succinic decarboxylase system is most active after 36 to 38 hours' incubation under the conditions stated, and shows greatest activity at pH 5.1 to 5.2. It is over 90 per cent destroyed by drying, and is approximately 90 per cent inhibited by 0.3 M malonate.

The production of propionic acid from pyruvic acid is almost completely inhibited by the presence of 0.3 M malonate.

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ELECTROKINETICAL STUDIES ON BACTERIAL SURFACES

II. STUDIES ON SURFACE LIPIDS, AMPHOTERIC MATERIAL, AND SOME OTHER SURFACE PROPERTIES

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The cytological and immunological data on the chemical composition of the cell wall and slime layer of the bacterial cell have been discussed by Knaysi (1944) and Dubos (1945). The methods of microelectrophoresis have also been used in studying surface phenomena of bacterial cells by Abramson (1934), Abramson, Moyer, and Gorin (1942), Dyar and Ordal (1946), and others. These data provide much useful information concerning the properties of the cell wall, but, unfortunately, there is little known concerning its chemical composition. Likewise, although the capsular material of some bacteria is relatively easy to separate from the rest of the cell and has been identified as consisting of high molecular weight polysaccharides, often with acetyl and amino groups (Dubos, 1945) or of polypeptides (Hanby and Rydon, 1946), in many cases its chemical composition and origin are both unknown. However, there is good evidence that the outer surfaces of bacteria differ widely, and that most are complex in composition.

Some surface constituents have an electrophoretic response distinctive enough to detect even in a complex surface. This principle has been nicely demonstrated by Bradbury and Jordan (1942), whose studies on the effects of *p*-aminobenzoic acid and related compounds on the mobility of *Escherichia coli* indicated that the drugs associated with the cells through their aromatic amino groups, and by Cohen (1945), who detected imidazole and amino groups at the surface of a species of *Proteus* by substitution with benzene sulfonyl chloride. Response to pH reveals even small amounts of amphoteric material in a surface although it would not be expected to distinguish kinds of amphoteric substances. The experiments of Dyar and Ordal (1946) on bacteria and on known surfaces indicated that lipid and perhaps other surface substances may be distinguished by their electrophoretic behavior in the presence of surface-active agents.

Such specific electrophoretic techniques coupled with other appropriate procedures seem to offer great promise in studying the chemical nature of the outer membranes of bacterial cells. By a careful choice of reagents to remove certain surface materials, for instance by the use of solvents or enzymes, some of the substances occurring in a complex surface might be identified with considerable certainty, and at the same time their removal should simplify the study of the remaining surface. The success of enzyme treatments is illustrated by the

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work of Avery and Dubos (1931), who showed that an enzyme extracted from a soil bacillus depolymerized the capsule of living, type III pneumococci.

In the present study we have attempted to find out more about the surface of bacterial cells. We have extended some of the electrophoretic procedures of characterizing cell surfaces developed by Dyar and Ordal (1946) by combining them with suitable chemical and enzymatic treatments to "strip off" parts of the surface.

The methods were essentially the same as those described by Dyar and Ordal (1946). The data were calculated as electrophoretic mobility expressed in microns per second per volt per centimeter, corrected to 25 C (Moyer, 1936).

The most intensive studies were made on *Micrococcus aureus* because in preliminary experiments its surface was found to contain several components that could be detected easily. *Mycobacterium smegmatis* and several species of *Bacillus* were also investigated. In most of the experiments the medium was 0.5 per cent peptone and 0.25 per cent yeast extract broth to which glucose or other substances were added. The cultures were usually grown for 12 to 24 hours at 30 C on a shaking machine. *Mycobacterium smegmatis* was grown at 37 C without shaking. Other conditions of growth as well as the preparation of some bacterial products are described later.

All experiments were performed in buffer solutions of 0.02 ionic strength. Phosphate buffers were used for pH 6 to 8 (Cohn, 1927), acetate buffers for pH 4 to 5 (Cohn, Heyroth, and Menkin, 1928), and HCl-KCl buffers for pH 2 to 3.

A number of surface-active agents were used in this investigation. These included the sodium salts of dodecyl, tetradecyl, and hexadecyl sulfonic acid, which were found to be more significant in studying lipids than sodium tetradecyl sulfate (2-methyl-7-ethyl undecanol-4-sulfate) used in the earlier experiments on bacterial surfaces, and cetyl pyridinium chloride. Highly purified preparations of sodium dodecyl, tetradecyl, and hexadecyl sulfonate were kindly supplied by Dr. E. J. Ordal, and the cetyl pyridinium chloride was obtained from Dr. Otto Rahn.

Lipase was partially purified by the method of Glick and King (1933). Dried pancreas powder (Merck) was extracted with 10 per cent NaCl and precipitated several times by saturation with $MgSO_4$. The fraction soluble in phosphate buffer at pH 8 was used. This solution was actively lipolytic when tested by Sumner and Somers' (1944) qualitative test employing an emulsion of castor oil as the substrate. Purified trypsin was prepared from pancreatin powder by reversible heat denaturation as described by Anson and Mirsky (1933).

Micrococcus aureus

Surface lipids. The exploratory experiments of Dyar and Ordal (1946) showed that the electrophoretic mobility of *Micrococcus aureus* and some other bacteria was greater when the cells were suspended in buffer solutions containing

anionic surface-active agents than when in buffer alone. With *Micrococcus aureus* this result was found to be much more marked when the cells were grown in aerated glycerol broth than when grown in plain broth. Larson and Larson (1922) had shown that the total ether-acetone extracts of *Micrococcus aureus* were increased fivefold by growing the cells in 3 per cent glycerol broth. Furthermore, Ordal and Dyar (unpublished) found that droplets of a hydrocarbon and of lipids also acquired a greater negative charge in solutions of anionic surface-active agents, presumably due to solution of the hydrocarbon ends of the surface-active molecules in their surface with the negatively charged polar groups oriented outward. These observations led them to suggest that the material in the cell surface responsible for the increase might be lipid. The first step in the present study was concerned with this possibility.

Evidence for the occurrence of lipid in the surface of cells was sought by comparing the electrophoretic behavior of cells grown in glucose broth with that of lipid extracted with alcohol from lyophilized cells of the same culture. It seemed likely that lipid extracted from cells themselves might come the closest to being like that at the cell surface. The alcohol extract was concentrated under vacuum and extracted with ether. An emulsion of lipid droplets was made by forcing the ether solution through a fine hypodermic needle into water and bubbling air through to expel the solvent. The results of the experiment are plotted in figure 1 and show that in the presence of the anionic surface-active agents the cells respond electrophoretically much like the lipid. The initial charge in buffer solution is not the same because other materials that influence it must be present in the bacterial surface. However, the character of the mobility-concentration curves in respect to increase in mobility, the concentrations at which the increase occurs, and the attainment of a maximum value are very similar. A striking fact is that the cells are not killed by these conditions.

The electrophoretic behavior of a variety of materials treated with surface-active agents has been studied by Ordal and Dyar (unpublished). They found that the negative mobility of mineral oil and crystals of sodium tetradecyl sulfonate, like the bacterial lipid, increased to a maximum value of 6.4 microns per second per volt per centimeter in buffered sodium tetradecyl sulfonate solutions. On the other hand, the mobility of surfaces such as egg albumin, cellulose, and pyrex remained constant with increasing concentrations of anionic surface-active agents in neutral or slightly alkaline buffers. When the pH was adjusted closer to its isoelectric point, the mobility of egg albumin in anionic surface-active agent solutions did increase but did not become greater than the maximum negative charge in buffer alone.

To obtain definite evidence of the electrophoretic behavior of other compounds of bacterial origin, a nucleoprotein and a polysaccharide were investigated. The nucleoprotein was prepared by Dr. Willard Schmidt from a ground, acetone preparation of *Streptococcus* s.b.e. Its purity was shown by a single, high adsorption peak at 2,590 A. The mobility of the nucleoprotein, shown in table 1, remains constant like egg albumin at pH 8 in the presence of sodium tetradecyl sulfonate. When the pH was lowered, it increased in the sodium tetradecyl

sulfonate solutions. The mobility-pH curve of the nucleoprotein had been found to level off above pH 6.9, so at pH 8 the maximum number of negative groups must have been exposed on the protein surface. The increase in negative charge at the lower pH may have resulted from neutralization of some positive polar groups by the negatively charged surface-active molecules.
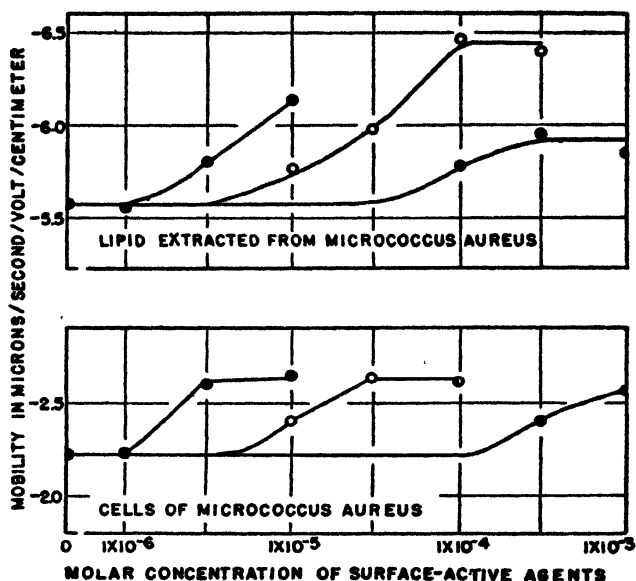
Figure 1. Mobility-concentration curves of *Micrococcus aureus* and of lipid extracted from *Micrococcus*, treated with sodium hexadecyl sulfonate ●————●, sodium tetradecyl sulfonate ○————○, and sodium dodecyl sulfonate ☉————☉.

TABLE 1

Mobility of nucleoprotein and dextran in solutions containing sodium tetradecyl sulfonate

	MOLAR CONCENTRATION OF SODIUM TETRADECYL SULFONATE		
	0	3×10^{-5}	1×10^{-4}
Nucleoprotein, pH 8..................	−3.0		−3.0
Nucleoprotein, pH 4.............................	−1.1	−1.0	−2.0
Dextran.................................	0.0	0.0	0.0

An insoluble polysaccharide was prepared from a culture of *Streptococcus* s.b.e. grown in sucrose broth as described by Niven, Kiziuta, and White (1946). This polysaccharide was identified chemically by Niven *et al.* and serologically by Hehre and Neill (1946) as a dextran. The electrophoretic results for the dextran are shown in table 1. Like cellulose, it had zero mobility in both buffer and sodium tetradecyl sulfonate solutions. Some cells remained embedded in the polysaccharide, which fact may account for the trace of nitrogen reported

by Niven *et al.* in the dextran. Other cells which did not remain embedded in the capsular material had a negative mobility. With *Streptococcus* s.b.e., the suggestion which has sometimes been made that the bacterial capsule is a modification of the substance of the cell wall does not appear to be likely.

From these results it seems that lipid treated with anionic surface-active agents may indeed be distinguished from other types of surfaces by its electrophoretic behavior. It is evident, however, that certain precautions are essential in drawing such a conclusion. For example, if amphoteric groups are present, the buffer must have a pH such that the negative charge of the surface is maximum.

There apparently is lipid at the surface of *Micrococcus aureus*, so we tried to remove it with an enzyme. Live cells were exposed to lipase in phosphate buffer at pH 8. After treatment, the cells were washed three times with buffer, and the electrophoretic behavior was determined. These cells were still viable as determined by comparing plate counts made on a suspension of cells with and without the enzyme treatment; no difference was found between them. The electrophoretic results of one such experiment are recorded in table 2, which

TABLE 2

Mobility of Micrococcus aureus before and after treatment with lipase

	MOLAR CONCENTRATION OF SODIUM TETRADECYL SULFONATE			
	0	1×10^{-5}	3×10^{-5}	1×10^{-4}
Control cells..........	−3.2	−3.3	−3.4	−3.4
Cells exposed to lipase for 90 minutes.	−2.9		−2.9	−2.9

shows that the mobility of cells treated with lipase no longer increases in the solutions containing sodium tetradecyl sulfonate. Apparently all detectable lipid has been removed.

When cells exposed to lipase were sampled at several time intervals, a gradual change in the increase in charge or the amount of lipid was found between the control and the treated cells. The lipase was effective on both live and heat-killed cells; both were still gram-positive after the enzyme treatment. To check the possibility that the lipid was not actually removed by the lipase but only masked by adsorption of the enzyme, solutions of trypsin and mold amylase, neither of which contained lipase, were allowed to act on cells. Neither altered the characteristic increase in mobility in the presence of sodium tetradecyl sulfonate.

After a suspension of live cells had been lipase-treated, small amounts of fatty acids were recovered by acidifying the supernatant and extracting with ether. Approximately 4 mg of fatty acids were recovered from live cells having a dry weight of 200 mg. In contrast, Ballentine and Parpart (1940) concluded that pancreatic lipase split a fatty acid molecule from each phospholipid molecule present in the red blood cell surface but did not displace the fatty acids from the surface.

The production of surface lipid, as indicated by the electrophoretic response, was found to be influenced by growth conditions. A barely detectable increase in charge was found for cells grown in peptone yeast extract broth without aeration. A slightly greater increase was found for cells grown in this medium with aeration or in 3 per cent glycerol broth with aeration. The greatest increase was evident for cells grown with 0.5 per cent glucose and moderate aeration. In one such culture the cells had a negative mobility of 2.3 microns per second per volt per centimeter in buffer, 2.5 in 1×10^{-5} M, and 2.7 in 3×10^{-5} and 1×10^{-4} M sodium tetradecyl sulfonate. These cells had lipid in the surface during the logarithmic growth, at the peak of the logarithmic growth, and during the death phases of the growth cycle. When the acids formed by the fermentation of the glucose were neutralized at frequent intervals, the mobility of cells in both buffer and in sodium tetradecyl sulfonate solutions was 2.4, indicating that no surface lipid was formed under these conditions. When such a culture was again permitted to lower the pH, surface lipid accumulated.

It is known from the experiments of Smedley-Maclean (1922), Starkey (1946), and others that the fat occurring in yeast cells is very incompletely extracted by ether or even boiling alcohol. Only after the cell structure is destroyed by acid hydrolysis is all the fat extractable. Anderson and Roberts (1930) showed that this is also true of tubercle bacilli, and in a study of *Bacillus cereus*, which we used because of its large size, we obtained parallel results. The fatty inclusions and the cytoplasmic membrane of *Bacillus cereus* were found to stain with Sudan black B after both ether and hot alcohol extraction of dried cells, although the intensity of staining was reduced slightly after each treatment. Much of the lipid in the cytoplasm and cytoplasmic membrane of cells must be linked with other constituents. To discover if the same was true of surface lipid, cells of *Micrococcus aureus* grown in glucose broth were lyophilized and extracted in a Soxhlet apparatus with petroleum ether for 20 hours. The electrophoretic results of this experiment, in which three of the anionic surface-active agents were used as indicators, are shown in figure 2. The mobility-concentration curves for the ether-extracted cells are quite different from the dried cell control, as though the surface had been altered by the extraction, but apparently the lipid has not been removed. The same cells were then extracted with hot ethyl alcohol for 17 hours. These results are also plotted in figure 2 and indicate that the lipid is partly removed by this procedure. This experiment suggests that the lipid must be thoroughly bound to the cell surface even though it is removed by lipase without altering the viability of the cells.

Surface amphoteric material. In the first experiments on *Micrococcus aureus* it was noted that the mobility of cells poor in surface lipid was relatively independent of pH, and, conversely, the mobility of cells grown under conditions favoring surface lipid changed considerably with pH. For example, cells grown in plain broth without aeration migrated with a negative mobility of 2.56 microns per second per volt per centimeter at pH 8 and 2.28 at pH 4, whereas cells grown in glucose broth with aeration had a mobility of 2.65 at pH 8 and only 1.05 at pH 4. However, cells grown in glucose broth which was kept neu-

tralized had no surface lipid but did change charge with pH.   Such a culture had a mobility of 2.40 at pH 8 and 1.08 at pH 4.   Hence, in *Micrococcus aureus* there is clearly an ionizable, surface component, distinct from the lipid, the occurrence of which is dependent on conditions of growth and that can be detected electrophoretically.

Several experiments were planned to discover more about this material. The mobility-pH curve of cells grown in glucose broth is plotted in figure 3 and shows that the ionizable material is amphoteric with the charge reversed at about pH 3.   The isoelectric point of the amphoteric substance is not necessarily so low as pH 3 because the presence of other surface materials of course
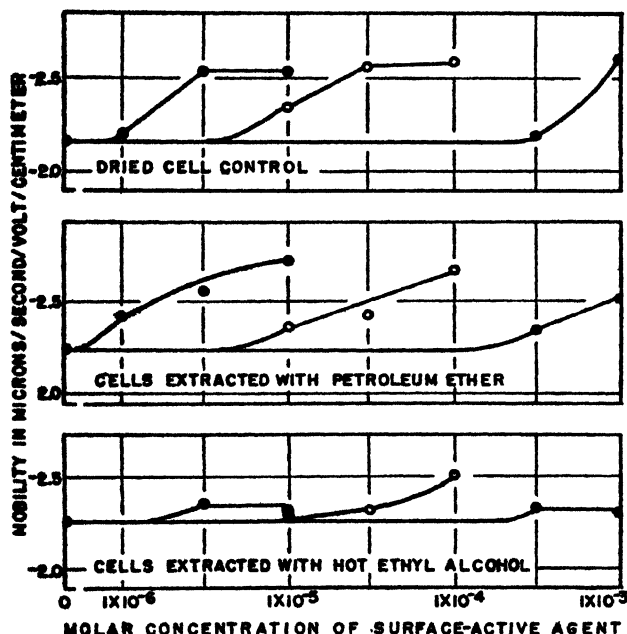
Figure 3. Mobility-concentration curves of *Micrococcus aureus* extracted with fat solvents, treated with sodium hexadecyl sulfonate ●————●, sodium tetradecyl sulfonate O————O, and sodium dodecyl sulfonate ☉————☉.

influences the mobility in any buffer solution.   If it were of protein nature, a proteolytic enzyme might be expected to remove it.   Lancefield (1943) found that pepsin, trypsin, and chymotrypsin readily removed the protein M antigen from the surface of living, group A, hemolytic streptococci.

In the experiment shown in figure 3, live cells of *Micrococcus aureus* grown in glucose broth were exposed for several hours to trypsin solution buffered at pH 8 and were then washed thoroughly.   The electrophoretic results show that the amphoteric material has been removed by this procedure.   Removal of the amphoteric material did not expose a greater amount of lipid.   It was determined, as in the case of lipase treatment, that the enzyme acts on both heat-killed

and live cells, the gram reaction is not altered by the enzyme action, and live cells are still viable after removal of the amphoteric surface material by trypsin.

Other surface properties. Other compounds than lipids and amphoteric material probably occur at the surface of *Micrococcus aureus* too, but, owing either to quantity or kind, have not been detected by the methods used. In this regard it should be noted that Julianelle and Wieghard (1934) showed that micrococci may be separated into two types on the basis of serologically different polysaccharides. However, it was not possible to differentiate strains
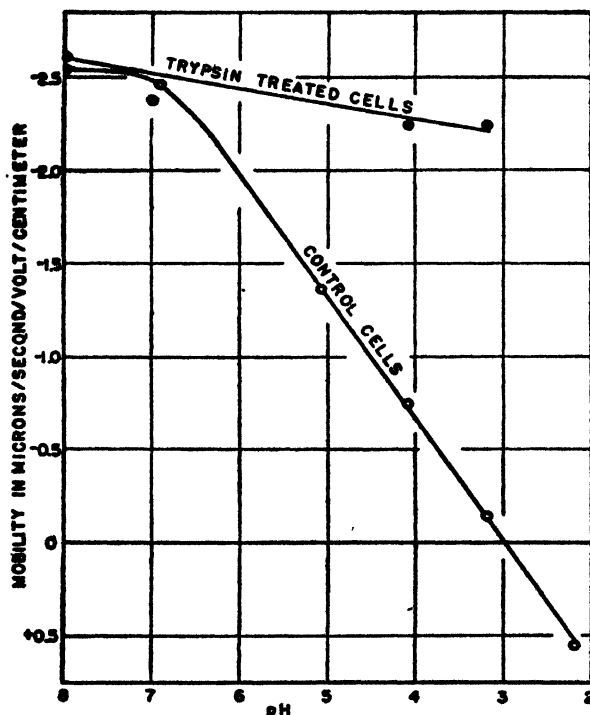
Figure 3. Mobility-pH curves of *Micrococcus aureus* with and without trypsin treatment.

by agglutination, so presumably the specific carbohydrates were not exposed at the surface.

As noted in connection with other experiments, the electrophoretic mobility both of cells treated with trypsin and lipase and of cells grown in such a way as to minimize the formation of amphoteric material and lipid was found to be little influenced by pH or anionic surface-active agents. The mobility-concentration curves with a cationic surface-active agent were also found to be similar but not unique enough to help identify the composition of this part of the cell membrane or cell wall. The indication, from such negative evidence, is that it consists of some kind of carbohydrate, so a comparison to cellulose and the insoluble dextran synthesized by *Streptococcus* s.b.e. was especially interest-

ing. Both the carbohydrates had zero mobility under the conditions of these experiments, whereas the cell surfaces have a definitely negative charge. They may be carbohydrate unlike these, or other substances responsible for the negative charge may still be present.
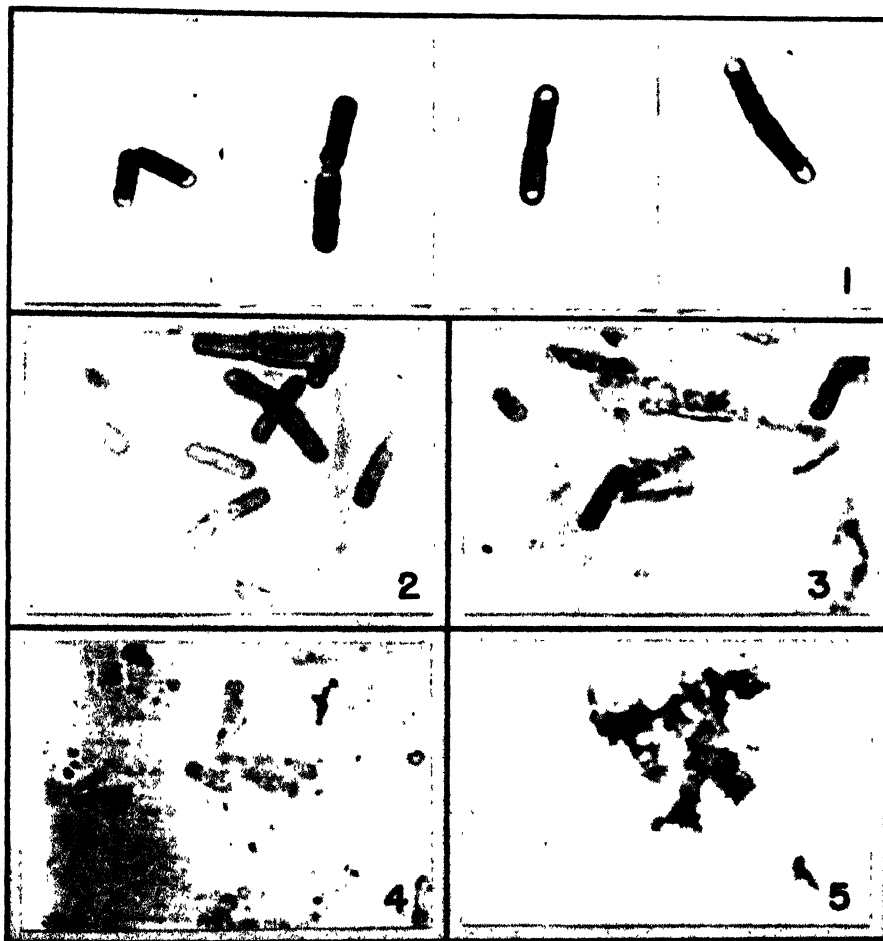
Figure 4. Cells of *Bacillus cereus* hydrolyzed with N HCl at 100 C for (1) 0, (2) 10, (3) 20, (4) 30, and (5) 40 minutes and stained with a cell wall stain (X 1,500).

The complexity of compounds such as carbohydrates is somewhat proportional to the intensity of acid treatment required to hydrolyze them. To determine the effect of acid hydrolysis on the cell wall, it was first necessary to have a cell wall stain capable of revealing it after such a treatment. Neither Knaysi's (1941) nor Robinow's (1945) cell wall stain seemed completely adaptable for this purpose, so a new one was devised. In this stain the cell surface is positively charged by treatment with a cationic surface-active agent (cetyl py-

ridinium chloride) and is then stained with an acid dye (Congo red). The details of the procedure have been reported elsewhere (Dyar, 1947). Because of its size, *Bacillus cereus* was used in working out the method, which was then applied to *Micrococcus aureus*.

The cell walls of *Bacillus cereus*, stained by this method, were still clearly visible after relatively short exposure to hot acid, but stained paler and paler as hydrolysis proceeded until only cell fragments remained. This is illustrated in figure 4 by photomicrographs of cells hydrolyzed for 0, 10, 20, 30, and 40 minutes and then stained with the cell wall stain. Because the staining depends on the reaction of an acid dye with a positively charged cell surface, the paler stain could result from a less positive charge acquired by the partially hydrolyzed membrane when treated with the cationic mordant. To check this possibility, the mobility of hydrolyzed cells of *Bacillus cereus* treated with cetyl pyridinium chloride was measured. The positive mobility of cells exposed to N HCl at the temperature of boiling water for 0, 10, 20, and 30 minutes and then neutralized, washed, and suspended in a buffered 1×10^{-3} M solution of cetyl pyridinium chloride was, respectively, 0.5, 1.0, 1.0, and 1.1 microns per second per volt per centimeter. Therefore, the reduced intensity with which the hydrolyzed cell walls stain is not dependent on a lessened positive charge but must result from the progressive removal of the wall.

Cell walls of *Micrococcus aureus* subjected to this treatment were still discrete after 10 minutes, but after 12 minutes almost all the cells were reduced to fragments. In this regard no difference was found between cells grown so as to be poor in surface lipid and amphoteric material and those from which these substances had been enzymatically removed. For comparison, the same treatment was applied to several other bacteria. The cell walls of *Bacillus mycoides* and *Bacillus subtilis* required 30 minutes, *Escherichia coli* 10 minutes, and spore membranes, in contrast, still persisted after several hours.

Other Bacteria

Surface lipids. *Bacillus cereus* and *Bacillus mycoides* were found to have no lipid at the surface. Microscopic observation of cells of *Bacillus cereus* grown in glucose broth with vigorous aeration revealed that the cytoplasm was filled with fatty inclusions. However, the negative mobility of these cells in both buffer solution and in buffer containing 1×10^{-4} M sodium tetradecyl sulfonate was 1.84 microns per second per volt per centimeter. This result is very different from that obtained with *Micrococcus aureus*.

Some of the techniques developed in studying surface lipids of *Micrococcus aureus* were also applied to *Mycobacterium smegmatis*. It was found that the same concentrations of the three anionic surface-active agents used for *Micrococcus aureus* did result in an increased mobility of the cells of *Mycobacterium smegmatis*. Cultures were grown in broth containing 1, 5, and 10 per cent glycerol. At intervals of several days the cultures were sampled, and the cells were washed with buffer and suspended, either as single cells or small clumps, by drawing the suspension in and out of a syringe fitted with a fine needle. The

mobility of individual cells was rather variable, and the amount of surface lipid varied considerably both with the age of the culture and with the amount of glycerol in the medium. Under the conditions of this experiment, cell growth was greatest in the 5 per cent glycerol broth, and after 9 days these cells were the most strongly acid-fast and had the maximum amount of lipid in the surface. Their negative mobility was 2.3 in buffer, 2.6 in 1×10^{-5} M, 3.1 in 3×10^{-5} M, and 2.8 in 1×10^{-4} M sodium tetradecyl sulfonate.

A suspension of cells from a culture grown for 10 days in 5 per cent glycerol broth was exposed to lipase for 3 hours. The lipid was not diminished at all. This would imply that the enzyme is ineffective on the lipid in *Mycobacterium smegmatis*.

Amphoteric material. The mobility of the different cultures of *Mycobacterium smegmatis* changed with pH, though there was some variation among cultures grown for several periods of time or in different media as shown in table 3. Cells grown in 5 per cent glycerol broth for 10 days and treated with trypsin for 3 hours had a negative mobility of 3.2 at pH 8, 2.0 at pH 5, and 0.5 at pH 4. The amphoteric portion of the surface of *Mycobacterium smegmatis* clearly does not serve as a substrate for trypsin.

TABLE 3

Mobility of different cultures of Mycobacterium smegatis at pH 4 and 8

	pH 8	pH 4
5 per cent glycerol, 9 days	−2.3	−0.7
5 per cent glycerol, 12 days	−3.0	−0.2
1 per cent glycerol, 9 days	−2.7	0.0

DISCUSSION AND SUMMARY

Electrophoretic techniques of characterizing cell surfaces have been combined with chemical and enzymatic treatments to remove parts of the cell surface. Both lipid and amphoteric material can be detected and removed from the surface of *Micrococcus aureus* by treatments which do not kill the cell.

Experiments on known substances showed that lipid droplets have a greater negative charge when suspended in neutral or slightly alkaline buffer solutions containing anionic surface-active agents than when in buffer alone. On the other hand, proteins, including crystalline egg albumin and a bacterial nucleoprotein, carbohydrates, including cellulose and an insoluble bacterial dextran, and inorganic particles do not. This distinctive response of lipids has been utilized to detect lipids at the surface of bacterial cells.

It was found with *Micrococcus aureus* that the increased charge in solutions containing anionic surface-active agents was small when the cells were grown in plain broth and much greater when grown in aerated glucose broth. The data suggest that the magnitude of the increase in mobility may provide a means of estimating semiquantitatively the amounts of the surface lipid. If a surface is

100 per cent lipid, it was shown that it attains a maximum negative mobility of 6.4 microns per second per volt per centimeter in a buffered sodium tetradecyl sulfonate solution.   In one culture of *Micrococcus aureus* grown in glucose broth, the cells had an initial mobility of 2.3, and if they were 100 per cent lipid, the mobility would be expected to increase to 6.4.   However, it increased to only 2.7, which is 10 per cent of the total possible increase.   Therefore, it seems possible that the lipid may occupy roughly 10 per cent of the surface area. Using the same calculation, cells of *Mycobacterium smegmatis* grown in 5 per cent glycerol broth for 9 days had an average of about 16 per cent of their surface occupied by lipid.

Not having model surfaces with a known area consisting of lipids, we have been unable to test this hypothesis, but it is at least useful for relative comparisons. The 10 per cent estimated by this method is the greatest amount that cells of *Micrococcus aureus* have been induced to form.   The surface lipid was not seen microscopically when cells were suspended in a saturated ethylene glycol solution of Sudan black B, but it may be distributed in such a way as to be below the resolving power of the microscope.

The amount of lipid in the surface of cells of both *Micrococcus aureus* and *Mycobacterium smegmatis* can be altered at will, within limits, by varying the conditions of growth.   Other bacteria such as some bacilli do not produce surface lipids even though the cytoplasm is full of fat.   Much of the lipid is removed from the surface of *Micrococcus aureus* by extraction with hot alcohol but not by prolonged extraction with ether.

The surface lipid can be removed from *Micrococcus aureus* by lipase without killing the cells, and small amounts of fatty acids may be recovered from the supernatant after the lipase treatment.   The action of pancreatic lipase is not specific enough to identify the lipid.   However, after being treated with trypsin, cells still have surface lipid but their charge does not change with pH.   This indicates that ionizable polar groups, such as occur in fatty acids and phospholipids, are either not free or not oriented outward.

Pancreatic lipase does not remove lipids from the surface of *Mycobacterium smegmatis*.   This clearly shows that the surface lipid of *Mycobacterium smegmatis* is not like that of *Micrococcus aureus*.   This might have been predicted from the extensive work of Anderson (1941, etc.) and his associates who have shown that the lipids extracted from cells of mycobacteria are of unusual and complex types. It is interesting to note that Kelsey (1939) found castor bean lipase to be ineffective on cholesterol esters under the same conditions that neutral fat was quantitatively hydrolyzed.   The lipids that occur at the surface of *Mycobacterium smegmatis* may be such combinations as esters of the higher alcohols, whereas those of *Micrococcus aureus* are probably much simpler.

Amphoteric material in the surface of *Micrococcus aureus*, distinct from the lipid, was detected by change in mobility with pH.   Like the lipid, its occurrence depends on the growth conditions, and it can be removed from living cells by treatment with trypsin.   Trypsin is active on rather a wide group of substrates including polypeptides and many proteins, so the use of this enzyme classifies

the amphoteric material only into this rather wide range of substances. It is possible that the use of other proteinases or peptidases might restrict this range. Trypsin was not effective in removing the polar groups at the surface of cells of *Mycobacterium smegmatis*. Clearly this amphoteric material is not among the substrates that trypsin is capable of attacking.

Some of the properties of the cell surface of *Micrococcus aureus* remaining after lipase and trypsin treatment were determined. They indicate that it may be of carbohydrate nature, but the methods have not been extended far enough to detect compounds such as carbohydrates with certainty. The intensity of acid hydrolysis necessary completely to break down the cell wall of several bacteria was determined.

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